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Exposure to benzene and childhood leukaemia: a pilot case-control study

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ABSTRACT

Objectives

Main purpose: to assess the feasibility of a measurement-based assessment of personal benzene exposure in case-control studies of paediatric cancer.

Additional aims: to identify the main sources of variability in personal exposure; to evaluate the performance of two benzene biomarkers; to verify the occurrence of participation bias; to check whether exposures to benzene and to 50 Hz magnetic fields were correlated, and might exert reciprocal confounding effects.

Design

Pilot case-control study of childhood leukaemia and exposure to benzene assessed by repeated seasonal weekly measurements in breathing zone air samples and outside the children's dwellings, with concurrent determinations of cotinine, *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) in urine.

Participants

Full-participation obtained from 46 cases and 60 controls, with low dropout rates before 4 repeats (11% and 17%); additional 23 cases and 80 controls allowed collection of outdoor air samples only.

Results

The average benzene concentration in personal and outdoor air samples was 3 $\mu g/m^3$ (SD 1.45) and 2.7 $\mu g/m^3$ (SD 1.41), respectively.

Personal exposure was strongly influenced by outdoor benzene concentrations, higher in the cold seasons than in warm seasons, and not affected by gender, age, area of residence, or caseness.

Urinary excretion of S-PMA and personal benzene exposure were well correlated.

Outdoor benzene levels were lower among participant controls compared to non-participants, but did not differ between participant and non-participant cases; the direction of the bias was found to depend on the cut-point chosen to distinguish exposed and unexposed.

Exposures to benzene and ELF-MF were positively correlated.

Conclusions

Repeated individual measurements are needed to account for the seasonal variability in benzene exposure, and have the additional advantage of increasing the study power. Measurement-based assessment of benzene exposure in studies of paediatric cancer, although financially and logistically demanding, appear feasible and acceptable to children and their parents.

Article focus

- Benzene is an established causative factor for acute non lymphocytic leukaemia, and there is limited evidence limited evidence for an association between exposure to this agent and other hematologic neoplasms including acute lymphocytic leukaemia. Exposure to benzene would increase the risk of leukaemia at relatively high levels of lifetime environmental exposure (≥120 ppb). While it seems unlikely that benzene is a major cause of leukaemia in the general population, children may represent a subpopulation with increased susceptibility. Available studies of benzene and childhood leukemia have provided inconsistent results, possibly due to the use of surrogate exposure proxies, and lack of analyses by leukaemia subtype. To get further insights on this topic, epidemiological studies based on objective estimates of environmental exposure to benzene have been recommended.
- Our pilot study was aimed at evaluating the logistic feasibility of an assessment of personal benzene exposure based on repeated individual measurements within a case-control study of childhood leukemia. Additional aims were: (i) to estimate the level of benzene exposure in children and assess if, and how much, exposure variability was affected by a number of putative determinants; (ii) to evaluate the performance of urinary levels of *t*-*t*-muconic acid (MA) and sulpho-phenylmercapturic acid (S-PMA) as benzene biomarkers in children; (iii) to assess the presence of participation bias (which occurs when adhesion to the study protocol is associated with both the level of exposure and the presence / absence of the disease); (iv) to determine whether exposures to benzene and to 50 Hz magnetic fields (ELF-MF) were correlated, so that they could exert reciprocal confounding effects in the analyses of their relationship with childhood leukemia.

Key messages

- Eligibility for inclusion was restricted to 108 cases and 194 matched controls, aged 2 to 12 years at the time of the survey. Full participation rates were low (cases 43%, controls 31%), but additional 21% of cases and 41% of controls accepted the outdoor monitoring. Adherence of full participants to the scheduled four seasonal repeats was very satisfactory (cases 89%, controls 83%).
- Personal exposure was strongly influenced by outdoor benzene concentrations, was higher in the cold seasons than in warm seasons, and was not affected by gender, age, area of residence, or caseness. Personal benzene exposure and urinary excretion of S-PMA (but not of

MA) were well correlated. Outdoor benzene levels were lower among participant controls compared to non-participants, but did not differ between participant and non-participant cases (a participation bias was indeed present). A positive association between exposures to benzene and ELF-MF was observed.

- Epidemiologic studies of paediatric cancer and estimates of environmental benzene exposure based on repeated seasonal measurements, although challenging, appear logistically feasible and acceptable to children and their parents.

Strengths and limitations

- To our knowledge, this is the first pilot study of childhood leukaemia and measured personal benzene exposure. Its also has the merit of having addressed a number of methodological problems besides logistic feasibility issues.
- Due to logistic reasons and resource constraints, the study size was very small. It must also be stressed that the expected greater accuracy of measurement-based exposures estimates, compared to surrogate exposure proxies, does not necessarily correspond to increased construct validity; this is especially true when measurements are used for retrospective post-diagnosis exposure assessments.

INTRODUCTION

Benzene is a ubiquitous air pollutant, that needs to be metabolized to become carcinogenic.[1- 2]

Benzene exposure and acute non lymphocytic leukaemia are causally related, while there is limited evidence for an association between exposure to this agent and acute or chronic lymphocytic leukaemia, multiple myeloma, and non-Hodgkin's lymphoma.[3]

Exposure to benzene would increase the risk of leukaemia at levels of ≥40 ppm-years of occupational cumulative exposure, equivalent to a lifetime (76 years) environmental exposure of ≥120 ppb.[4]

Due to the established carcinogenicity of benzene, WHO has not developed any guideline value for this chemical in air, while indicating that ambient benzene concentrations of 17, 1.7 and 0.17 $\mu g/m^3$ are associated with excess lifetime risks of leukaemia of 10^{-4}, 10^{-5} and 10^{-6}, respectively.[5-6]

While it seems unlikely that benzene is a major cause of leukaemia in the general population exposed in the ppb range, children may represent a subpopulation with increased susceptibility on intake or on key pharmacokinetic / pharmacodynamic processes.[1, 3]

Childhood leukaemias have distinctive features compared to leukaemias in adults. In precursor B cell acute lymphoblastic leukaemia (pre-B ALL) and some cases of acute myeloid leukaemia (AML), a first initiating genetic event has been shown to occur *in utero*, at a rate of up to 1% (for TEL-AML1 translocations in pre-B ALL). Further genetic changes are required to create a malignant clone. Ionizing radiation, benzene, alkylators and topoisomerase II inhibitors are among the few confirmed environmental risk factors for AML, while delayed, dysregulated responses to common infections are likely to play a major role in the conversion of pre-leukemic clones into overt ALL.[7]

Findings from available studies of benzene and childhood leukaemia are inconsistent, possibly due to the use of indirect estimates of exposure and lack of analyses by leukaemia subtype.[8]

To advance current understanding of benzene health effects and susceptibility, studies of paediatric cancers that include estimates of environmental exposure to benzene, rather than surrogate exposure indicators, have been recommended.[9]

Major challenges in pursuing this suggestion include the space- and time-variability of ambient benzene levels, the low exposure levels in children, and the inherent susceptibility of case-control studies (the design of choice for etiological studies of rare disease like childhood cancer) to selection and information bias.

We evaluated the logistic feasibility of an assessment of benzene exposure based on repeated seasonal weekly measurements in breathing zone air samples and outside the children's dwellings, with concurrent determinations of cotinine, *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) in urine, in a pilot investigation within an Italian case-control study on environmental risk factors for childhood leukaemia (SETIL).

Additional objectives of the pilot study were:

- to investigate the relationship between level personal exposure to benzene and putative determinants (atmospheric benzene, second-hand tobacco smoke, individual traits);
- to assess the performance of *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) as benzene biomarkers in children;
- to verify the occurrence of participation bias from differential adhesion to the benzene measurement study, and estimate the amount and direction of the distortion;

– to check whether exposures to benzene and to extremely low frequency magnetic fields (ELF-MF) were correlated, and might eventually exert reciprocal confounding effects on the relationship with childhood leukaemia.

METHODS

Study population

Incident cases of childhood leukaemia from 14 Italian regions, aged 0 to 10 years at diagnosis in 1998-2001, were eligible for enrolment in the SETIL study. Cases were ascertained through the national registry run by the Association of Paediatric Haematology and Oncology (AIEOP). Controls, matched to cases (2:1 ratio) on gender, date of birth, and region, were randomly selected from population lists. Information on several items concerning the children, their next-of-kin and dwellings, was collected by interview of parents. All interviewed families were invited to participate in a measurement study of indoor ELF-MF, while subsets of participants were asked to join two side-investigations, on exposure to gamma radiation and benzene, respectively.

Eligibility for the benzene pilot study was restricted to 108 childhood leukaemia cases from seven Italian provinces (Turin, Milan, Florence, Rome, Catania, Palermo, and Cagliari), diagnosed between July 2000 and December 2001, and 194 matched controls.

The study protocol was approved by the Piedmont Ethical Committee on 14 January 2002.

Sampling strategy and devices

Due to the high daily and seasonal variability of atmospheric benzene concentrations, the protocol called for four repeated seasonal one-week samplings of breathing zone air per child over one year ("personal" air samples), with concurrent collection of urine samples and atmospheric air samples in proximity of the children's homes ("outdoor" air samples).

Outdoor air sampling would also be performed, with an identical strategy, near the homes of all eligible non-participants.

To study the day-to-day variability in exposure, 24-h repeated personal and indoor samples during four season-specific weeks would be collected from a subset of children and related homes.

Personal air samples were collected by passive samplers (Radiello® radial symmetry diffusive sampler) worn by the child during the day and placed at the bedside at night.

Radiello® samplers were also used to collect outdoor air samples, placed near the entrance of the dwellings (within 1 meter), at a vertical distance from the ground suitable to avoid infringements (2-2.5 m), stored in a plastic case to avoid rain or snow.

At retrieval, the adsorbing cartridges were removed from the diffusive bodies and placed into glass storage tubes. The ID code of the child, along with dates and times of sampling start and end, were recorded on self-adhesive labels stuck on the tubes. The cartridges were sent to a single laboratory (Fondazione Salvatore Maugeri, Padova) for the chemical analyses.

Daily urine samples (10 ml, from the last micturition before sleep) were collected for 7 subsequent days (70 ml per week) during each seasonal survey. The daily samples were pooled in one plastic vial, and kept in the freezer compartment of the home refrigerator until collection at the end of the week. The vials were transported to the local research centre in cool bags, and stored at –5 °C until delivery (packed in dry ice and usually in 2 weeks) to the laboratory (Fondazione Salvatore Maugeri, Pavia).

Field work began between March 2002 and January 2003, and ended in October 2003 - July 2004, depending on the local research centre.

Chemical determinations

Benzene concentrations in air sample were determined by an automated thermal desorber (ATD400, Perkin Elmer) coupled to a capillary gas-chromatography system (Autosystem XL, Perkin Elmer). The expanded uncertainty of the method, in the range 2.4 to 14.3 μg/m^3, was shown to be 18%.[10] The limits of detection and quantification, over 1 week exposure, are 0.05 μg/m^3 and 0.1 μg/m^3.

The urine analyses were performed using a high pressure liquid chromatography system (Alliance 2690, Waters) equipped with a spectrometric (SM) detector (ZQ, Waters) following a preliminary step of purification of the samples on pre-activated solid phase extraction (SPE) cartridges. The limit of detection (LOD), coefficient of variation (CV) and accuracy of the method were: LOD = 1 μg/L, CV % = (1.22)-(1.10), accuracy % = (- 2.39)-(3.36) for S-PMA; LOD = 20 μg/L, CV % = (1.33)-(1.06), accuracy % = (- 2.18)-(3.27) for MA; LOD = 1 μg/L, CV % = (1.25)-(1.09), accuracy % = (-2.29)-(3.33) for cotinine.

Further details are provided in Appendix 1.

The chemical determinations were completed by May 2005.

Statistical analyses

Measurements below the chemical-specific detection limits were assigned half such values and included in the analyses.

The relationships between personal exposure to benzene and putative determinants (as well as between urinary excretion of benzene metabolites, benzene intake, and other covariates) were assessed by generalized least squares (GLS) models for repeated measurements (STATA v. 11, xtreg procedure). The GLS model is: $y_{it} = \alpha + X_{it}B + u_{it} + e_{it}$, where i (1 to n) is the number of observations collected at time t (1 to 4) and u_{it} and e_{it} are the error components.

As concentrations of benzene and urinary analytes were log-normally distributed, we always included in the models log-transformed dependent variables.

We used the odds ratio (OR), calculated from generalized estimating equations (GEE) for repeated individual measurements (STATA v. 11, procedure xtgee), to estimate the association between benzene exposure and dichotomous variables such as case-control or participation status. The general equation of the GEE model is $g\{E(y_j)\}=x_j\beta$, where g is the link function, herein a logit function.

We calculated a participation bias factor following the method suggested by Greenland [bias factor = $(S_{1a}*S_{0b})/(S_{0a}*S_{1b})$], where S_{1a}, S_{0a}, S_{1b}, and S_{0b} denote the probabilities of selection (i.e. full participation in the benzene study) for exposed cases, unexposed cases, exposed controls, and unexposed controls.[11] When the bias factor equals 1, there is no bias, when it is above or below 1 the true OR will be biased respectively upward or downward by the magnitude of this factor.

Multiple regression models were used to analyze the relation between estimated exposures to benzene and ELF-MF.

RESULTS

Participation and sampling outcome

Out of 108 cases and 194 controls eligible for inclusion, 46 cases and 60 controls (43% and 31%) agreed to take full part in the benzene side-study (Figure 1).

In addition, the parents of 23 cases and 80 controls who refused the personal exposure assessment accepted the outdoor monitoring (partial participation = 21% and 41%).

Altogether 1467 air samples were collected. A small percentage (2%) were lost during monitoring (22 samplers stolen, 2 sampler plates broken, 3 cartridges lost), transport (8 missing labels) or

chemical analysis (2 cartridges broken on arrival at the laboratory; 1 sample lost due to equipment failure).

Benzene measurements from the day-to-day variability sub-study (19% of the total) could not be used because only four control children accepted the 24-h sampling scheme, and were replaced by the calculated weekly averages.

A further 20% of benzene measurements was removed from the data-set due to lack of compliance with the study protocol (indoor samples collected in place of the personal ones from children refusing to wear the sampler; time-or place-mismatch of personal and outdoor samples; "orphan" personal or outdoor samples; duplicate season-specific measurements; non-participants replaced with children ineligible for the benzene side-study].

For the same reasons, 107 out of 417 chemical determinations in urine (26%) were discarded.

Three cases and 5 controls were excluded from one or more analyses due to lack of complete measurement sets in all seasonal series and, although 89% and 83% of full-participant cases and controls did adhere to all four seasonal surveys, only 37% and 43% of them had four repeated analyzable observations.

Personal characteristics of the children

The families of cases participating in full to the benzene study had been interviewed on average 1.3 years (SD 0.47) after the date of diagnosis, and the control-families 1.5 years (SD 0.46) after the corresponding reference date. The delay between diagnosis and the first series of benzene measurements was 2 years (SD 0.53) for both cases and controls.

Cases and controls were comparable in terms of gender, age, and father's attained educational level (Table 1). A higher proportion of controls than cases had both parents smoking, and control-mothers were more educated than case-mothers. There were similar proportions of only children

in the case and control groups, while firstborn children were more frequent among controls than cases. Early schooling (attendance of crèche) was more common in cases than in controls. At the time of the benzene survey, most children were still living in the home occupied at birth or in the house they moved into after birth but before the date of diagnosis (cases 95%; controls 91%).

Level, variability, and determinants of personal exposure to benzene

The analyses of level, variability and determinants of personal exposure to benzene were based on 43 cases (39 ALL and 4 AML) and 56 controls, with 261 valid pairs of benzene concentrations in breathing zone and outdoor air (110 from cases and 151 from controls). A large proportion of these children (35%) had a single pair of concurrent measurements, unevenly distributed by season, with a disproportionally high number of summer samples (30 out of 35, all but one from a single centre).

The distributions, overall and by season, of benzene concentrations in personal and outdoor air samples, and of cotinine, MA and S-PMA in urine are described in Table 2.

Personal exposure to benzene was log-normally distributed (Shapiro-Wilk test = 0.938, $p<0.001$), and the mean benzene level over the individual yearly averages was 3 $\mu g/m^3$ (0.92 ppb).

The distribution of benzene outdoor concentration was skewed to the left in all seasons and the yearly averages were log-normally distributed as well (Shapiro-Wilk test = 0.948, $p = 0.001$); the average yearly benzene level near the children's homes was 2.7 $\mu g/m^3$ (0.83 ppb).

Both outdoor benzene concentrations and personal exposure levels were higher in the cold seasons (autumn-winter) than in the warm ones (spring-summer).

The European limit for benzene in air (5 $\mu g/m^3$) was exceeded by 5% of the yearly average outdoor concentrations, and by 8% of the yearly average levels in breathing zone air samples. A large

proportion of autumn and winter measurements were above 5 μg/m^3 (35% and 25% outdoor; 26% and 30% of the personal exposure estimates).

Cases and controls had similar levels of personal exposure to benzene: the leukaemia OR for a unit increase (1 μg/m^3) in personal benzene exposure was 0.93 (95% CI 0.77-1.13) adjusting for gender, age at the benzene survey (2-4; 4-6; 6-12 years), cotinine in urine (μg/g creatinine), season, and province of residence (Turin; Milan; Florence - Rome; Catania - Palermo - Cagliari).

A similar lack of association was found between the odd of disease and benzene concentration outside the children's homes [OR 0.94 (95% CI 0.80-1.09)], controlling for gender, age, smoking habits of the parents at the interview (non-smokers, mother or father smoking; both parents smoking), season, and province of residence.

Further adjustment for birth order and age at first schooling had no material effect on the observed leukaemia-benzene relationship [personal exposure: OR 0.92 (95% CI 0.75-1.13); outdoor benzene: OR 0.95 (95% CI 0.81-1.13)].

As cases and controls had comparable levels of benzene exposure, we carried out the analyses illustrated in the forthcoming paragraphs on the whole data-set, although always controlling for caseness.

Urinary cotinine concentration (μg/g of creatinine) was higher in children of smoking parents compared to children of non-smokers, and children with both parents smoking excreted a larger amount of cotinine than children with one parent smoking (Appendix Table A). Cotinine levels were higher in winter than in other seasons, and higher in children from central and southern Italy (Florence, Rome, Palermo, Catania, Cagliari) than in children from northern provinces (Turin and Milan). The high between- *vs* within-subject R^2 ratio is worth noting.

Personal benzene exposure was strongly influenced by outdoor benzene concentrations (Table 3-A), and apparently not affected by gender or age; the season showed a modifying effect, with increasing levels of personal exposure during autumn and winter; the fraction of variability explained by the model was higher for the within-subject component than for the between-subject one.

Exposure to second-hand tobacco smoke (estimated by cotinine excretion or by parental smoking habits) showed a trivial influence on personal exposure to benzene. The inclusion of urinary cotinine (μg/g creatinine) in the model described in Table 3-A, slightly decreased its goodness of fit [R^2 overall = 0.46; Wald χ^2=189.49; R^2 within = 0.55; R^2 between = 0.35; β (cotinine) = 0.012; 95% CI = -0.003; 0.03)]; an alternative model, including smoking habits of the parents, did not perform any better [R^2 overall = 0.46; Wald χ^2=216.44; R^2 within = 0.52; R^2 between = 0.39; β (one parent smoking) = 0.14; 95% CI = -0.02; 0.31; β (both parents smoking) = 0.17; 95% CI = -0.06; 0.39].

Children from central Italy (Florence and Rome) tended to have lower benzene concentrations in breathing zone air samples compared to residents in other provinces, all other things being equal (Table 3-A), possibly because of residual confounding from lack of samples collected in Rome other than in summer. We tried to verify this hypothesis by restricting the analyses to children with at least two series of measurements in different seasonal periods (cold and warm). The data-set reduced to 61 subjects (25 cases and 36 controls) and 220 pairs of personal-outdoor benzene measurements. Actually, children from Florence still showed (not significantly) lower levels of personal exposure to benzene (β = - 0.27; 95% CI = -0.56; 0.03; p =0.074) compared to children from Turin. In the restricted data-set, however, independent effects of both outdoor benzene and urinary cotinine levels on personal benzene exposure were observed (Table 3-B).

Benzene intake and urinary excretion of benzene metabolites

Ninety-eight children (43 cases and 55 controls) and 310 pairs of urine and breathing zone air measurements (138 from cases and 172 from controls) were available for the analyses of the urinary excretion of benzene metabolites (MA and S-PMA) in relation to personal exposure to benzene.

Urinary concentrations of S-PMA (ln µg/g creatinine) were related to personal exposure to benzene (Table 4, Model 1). Youngest children (2-4 years at the benzene survey) excreted higher level of S-PMA compared to children aged 6-12 years, all other conditions being equal, and urinary concentration of S-PMA were higher in samples collected during the cold seasons compared to spring samples. The model, however, explained just 19% of the overall S-PMA variability. In an alternative model, including outdoor benzene concentrations and urinary cotinine in place of personal benzene exposure, we also observed an effect of the nicotine biomarker on S-PMA excretion (Table 4, Model 2).

On the contrary, neither benzene concentrations in breathing zone air samples, nor outdoor benzene concentrations or cotinine levels explained the intra- and inter-individual variability in urinary levels of MA, controlling for gender, age, season, area of residence, and caseness (data not shown).

Bias due to differential participation

Available for the analysis of participation bias were 66 cases (43 full-participant and 23 partial-participant) and 136 controls (56 and 80), with 652 measurements of outdoor benzene concentrations (135 and 175 from full-participant cases and controls; 81 and 261 from partial-participant cases and controls).

Benzene concentrations near the homes of full-participant controls were significantly lower than those in proximity of partial-participants' dwellings (OR = 0.88; 95% CI 0.80-0.97), adjusting for

gender, age, season and place of residence, while there was no difference in ambient benzene levels between participant and non-participant cases (OR = 0.95; 95% CI 0.82-1.09). As participation in the study was also associated with the case-control status, assuming a causal association between exposure and disease, a selection bias might ensue. However, as parents of more exposed controls were less willing to accept to be interviewed, an upward distortion would be expected, which is at odds with the apparent lack of association between personal benzene exposure and leukaemia risk in the current study.

To the aim of the current analysis, personal exposure to benzene was dichotomized around the median (3.25 μg/m^3), the 75th percentile (4.34 μg/m^3) or 5 μg/m^3 (the current limit for airborne benzene in Europe). The amount and direction of bias were found to depend on the cut-point chosen (Appendix Table B), whereas no bias is expected when the exposure is categorized around the median (bias factor = 1.03), and biases in the opposite directions are predicted using cut-off at p75 and at 5 μg/m^3 (0.64 and 1.42, respectively).

Relationship between exposures to benzene and ELF-MF

Children with benzene and ELF-MF measurements made at the same house qualified for inclusion in the analysis of the relationship between estimated exposures to these agents. As only 35 cases and 46 controls met such criterion when benzene concentrations in breathing zone air samples were used as exposure indicator, we performed the analysis on 48 cases and 77 controls with place-comparable pairs of average yearly outdoor benzene concentration (μg/m^3) and 48 h TWAs of ELF-MF level in the child's bedroom (ln μT).

There was a positive association between estimated exposures to ELF-MF (dependent variable) and benzene (β = 0.177; 95% CI 0.06-0.29; p = 0.002); the multivariable regression model (including gender, age, province of residence, caseness, and participation in the benzene pilot study as covariates) explained 16% of the variability in the dependent variable [F (10, 114 df) =

2.13; p> F = 0.0271]. A steeper increase in ELF-MF level per unit increase in outdoor benzene concentration (β = 0.520; 95% CI 0.09-0.95; p = 0.019) was seen among the 81 children fully participating in the benzene pilot-study compared to the 44 partial-participants (Appendix Table C).

Similar results, with a more accentuated increase in indoor magnetic induction level per unit increase in outdoor benzene concentration [β= 0.272; 95% CI = 0.09-0.45; p(t) = 0.003; R^2 = 0.19], were observed in the restricted data-set of 86 children with ≥2 weekly samplings in alternate seasons.

DISCUSSION

We have carried out a pilot case-control study of childhood leukaemia and exposure to benzene assessed by repeated individual measurements made on average two years after diagnosis. The pilot study included side-investigations aimed at evaluating the performance of two biological indicators of benzene exposure in children, at estimating amount and direction of a possible participation bias, and at assessing the relation between estimated exposures to benzene and ELF magnetic fields.

Due to the relatively low incidence of childhood cancers (10-15 for 100,000 person-years in the 0-14 year range in most industrialized countries), the case-control approach is the design of choice for analytical epidemiologic studies about potential risk factors for these diseases. Such a study design, however, is inherently prone to measurement errors stemming from the retrospective reconstruction of the exposures of interest, and to differential participation leading to control samples not representative of the study base. Therefore, findings from observational epidemiologic studies of postulated determinants for childhood malignancies are often inconsistent and always require a cautious and thoughtful interpretation.[12]

Although based on small numbers, some of the findings from the current study have a certain factual and methodological interest.

Repeated samplings of breathing and outdoor air are indeed needed to account for the seasonal variability in environmental benzene levels.[13-14]

On average, children participating in the current study appear to experience mean yearly levels of personal exposure to benzene not exceeding the European guidelines (although 8% percent of the yearly mean levels were above 5 μg/m^3).

What we *a priori* considered the main sources of benzene exposure for children (ambient benzene levels and second-hand tobacco smoke) explained no more than half of the overall variability in personal exposure, which indicates the need to identify other sources of exposure particularly relevant, perhaps, during the cold seasons. In fact, in autumn-winter compared to spring-summer, we observed higher levels of personal exposure to benzene, of urinary cotinine and of S-PMA excretion, all other things being equal. These findings might be due to the lower ventilation rates in homes and schools during the cold seasons, to winter-specific sources of indoor benzene concentrations not considered in the current survey (e.g. fireplaces or other combustion sources), and/or to the seasonal variability in daily patterns of time spent in different micro-environments (e.g. within cars or buses).[15]

Some case-control studies have suggested an association between exposure to traffic density and childhood leukaemia;[16-19] however, negative findings have also been reported.[20-23] Positive associations between incidence of ALL in children and residential proximity to petrol stations were observed in three case–control studies.[21, 24-25] An increased risk of childhood leukaemia in relation to estimated exposure to benzene was observed in a small Italian study,[26] but not in a much larger case-control study carried out in Denmark and based on a sophisticated and validated exposure modelling.[27]

To our knowledge there is no previous study of childhood leukaemia and measured personal benzene exposure. Moreover, as only children aged 0 to 10 years at diagnosis were eligible for the SETIL study, the large majority of cases included in the current investigation were pre-B ALL.

Cases and controls did not differ in terms of exposure to benzene, estimated either by benzene level in personal air samples or through outdoor benzene concentration, but the interpretation of this finding is hampered by the retrospective exposure assessment and the low statistical power of this preliminary investigation. That notwithstanding, due to the design based on repeated individual observations, the risk estimates have quite narrow confidence intervals. Thus the findings from this pilot study, in accordance with the limited evidence for an association between exposure to benzene and ALL,[3-4] might also suggest that the levels of benzene exposure experienced by children living in Italian towns do not entail a detectable increase in the risk of ALL.

Current perspectives on the causes of childhood leukaemia increasingly point towards an etiologic role of altered patterns of infections and related immune stimulation during the first years of life, and one piece of supporting evidence is the consistent observation of an inverse association between ALL and day-care attendance.[28] Studies of childhood ALL and birth order, on the other hand, have provided inconsistent result.[29] Neither age at first schooling, nor birth order confounded the relation between childhood leukaemia and indicators of benzene exposure in the current study.

S-PMA concentration measured in repeated weekly samples of the last micturition before sleep was found to reflect personal exposure to benzene, although the available covariates explained a small fraction of the within- and between-subject variability of this benzene metabolite. This is a quite surprising result, considering that S-PMA is believed to represent less than 1% of urinary benzene metabolites for exposures to benzene at air concentrations between 0.1 and 10 ppm.[30]

Benzene exposure proved not able to explain the variability of MA urinary excretion observed in our children, consistent with findings from a previous Italian study.[31] The low statistical power of the study, the low level of benzene exposure, and the lack of adjustment for the confounding effect of dietary intake of sorbic acid (a common food additive), may explain this finding.[32]

Full-participation rates were higher among cases than controls. Notwithstanding the fairly satisfactory proportions of children with measured outdoor benzene concentrations (61% and 70% of eligible cases and controls), the degree of partial-participation was lower among non-participant cases (21%) than among non-participant controls (41%).

We observed a differential participation bias, which underscores the need to plan parallel bias analyses in any case-control study.[33] The dependence of the participation bias factor on the cut-point chosen to dichotomize the exposure variable is of methodological interest.

The positive association between the 48 h TWA of ELF-MF induction in the child's bedroom and the average yearly concentrations of outdoor benzene will need consideration in the interpretation of findings from the analyses of childhood leukaemia risk in relation to 50 Hz MF in the SETIL case-control study.

Incidental failures during sample collection, transport or chemical analysis accounted for a negligible proportion of lost air or urine samples. However, substantial percentages of chemical measurements could not be included in current analyses because of misunderstanding of the sampling protocol.

The day-to-day variability sub-study was clearly too demanding to be acceptable.

In conclusion, the current pilot study suggests that epidemiologic studies of childhood leukaemia risk and measurement-based estimates of exposure to benzene are challenging but logistically feasible (provided that the study protocol specifies every single sampling detail and nothing is

considered so obvious as to be omitted). Such an exposure assessment method could be considered by epidemiologists willing to involve in the "genome - exposome" approach to gain further insight into the relationship between benzene exposure and childhood leukaemia risk, with priority given to AML.[4, 34]

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COMPETING INTERESTS

None.

DATA SHARING STATEMENT

Additional explanatory material is available to everyone on request. The dataset is available to fellow researchers for further joint analyses, on request to the corresponding author, and pending approval by the co-authors.

CONTRIBUTORSHIP

Susanna Lagorio designed the study, planned the statistical analyses, and drafted the manuscript.
Daniela Ferrante carried out the statistical analyses.
Alessandra Ranucci was in charge of the data management, quality control and descriptive statical analyses.
Paolo Sacco and Sara Negri collaborated to the study design, and were responsible for the chemical analyses.
Roberto Rondelli, as manager of the AIEOP childhood leukaemia registry, performed the case ascertainment.
Santina Cannizzaro, Valeria Torregrossa, Pierluigi Cocco, Francesco Forastiere, Lucia Miligi, Luigi Bisanti, and Corrado Magnani were the principal investigators of the local centres collaborating to the benzene pilot study in the framework of the SETIL multicentre case-control study.
All the authors critically revised the early drafts, collaborated to the discussion of the study findings, and approved the final version of the manuscript.

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Table 1. Children included in the pilot study by selected characteristics

		Cases		Controls	
		N	%	N	%
Gender	Female	25	58	30	54
	Male	18	42	26	46
Age at the survey	[2,4) years	5	12	9	16
	[4,6) years	21	49	16	29
	[6,12] years	17	40	31	55
Residence*	Turin	7	16	9	16
	Milan	8	19	13	23
	Florence	3	7	5	9
	Rome	14	33	15	27
	Catania	3	7	5	9
	Palermo	4	9	6	11
	Cagliari	4	9	3	5
Parent smoking§	None	20	47	27	48
	One	16	37	18	32
	Both	4	9	11	20
	Missing	3	7	0	-
Father's education§	No qualification	-	-	1	2
	Primary school	17	40	21	38
	High school	17	40	24	43
	University degree	6	14	10	18
	Missing	3	7	-	-
Mother's education§	No qualification	-	-	-	-
	Primary school	19	44	17	30
	High school	15	35	26	46
	University degree	9	21	13	23
	Missing	-	-	-	-
Birth order§	Only child	10	23	12	21
	First born	10	23	20	36
	Second born or higher birth order	23	53	24	43
Age at first schooling§	No schooling yet	15	35	16	29
	<3 years (crèche)	14	33	9	16
	[3,6) years (preschool)	14	33	30	54
	[6-7] years (primary school)	0	-	1	2
Home at the time of the benzene survey^	Occupied since birth	28	65	39	70
	Moved into after birth & before diagnosis	13	30	12	21
	Moved into after diagnosis & before interview	1	2	5	9
	Moved into after interview	1	2	-	-
Total		43	100	56	100

*At the time of diagnosis or the corresponding reference date for controls; §Information reported at the interview; ^The ELF magnetic fields measurements, if the parents agreed, were made at the time of the interview.

Table 2. Benzene concentration in personal and outdoor air samples, and urine level of cotinine and benzene metabolites by season and overall

	Obs (#)	Mean	SD	G-mean	G-SD	Min	Percentiles			Max
Benzene in personal air samples (µg/m^3)							**p25**	**p50**	**p75**	
Spring	57	2.51	1.89	2.10	1.75	0.60	1.50	1.82	3.11	11.12
Summer	86	2.26	1.45	1.90	1.82	0.47	1.25	1.85	3.10	8.13
Autumn	62	4.31	2.60	3.73	1.57	0.92	2.939	3.70	5.17	18.47
Winter	56	4.04	1.78	3.67	1.73	1.55	2.34	4.00	5.24	9.03
Individual yearly averages	99	3.00	1.45	2.66	1.67	0.75	2.05	2.90	3.83	9.00
Benzene in outdoor air samples (µg/m^3)										
Spring	57	2.29	1.30	1.93	1.84	0.48	1.20	1.91	3.15	5.67
Summer	86	1.94	1.20	1.65	1.75	0.39	1.12	1.58	2.28	6.92
Autumn	62	3.99	2.58	3.05	1.92	0.08	1.93	3.42	5.63	11.18
Winter	56	3.80	1.86	3.25	2.35	0.15	2.40	3.66	5.20	8.31
Individual yearly averages	99	2.70	1.41	2.33	1.78	0.27	1.59	2.37	3.63	6.92
Cotinine (µg/ g creatinine)										
Spring	78	3.92	7.04	1.91	3.26	0.05	1.00	1.94	3.50	49.0
Summer	78	3.20	5.52	1.50	3.59	0.09	0.82	1.68	3.71	41.4
Autumn	76	4.54	8.51	1.92	3.92	0.05	1.20	1.93	4.30	48.7
Winter	74	4.36	7.38	2.32	3.01	0.10	1.20	2.30	4.80	53.5
Individual yearly averages	98	3.73	5.99	2.14	2.67	0.30	1.08	2.09	3.58	41.9
MA (µg/g creatinine)										
Spring	81	104.22	69.28	87.43	1.79	17.00	60.27	82.00	126.99	349.00
Summer	79	140.40	226.73	92.30	2.16	13.33	56.54	83.00	131.76	1680.00
Autumn	76	128.24	124.04	99.57	1.94	30.21	60.16	102.48	147.21	893.04
Winter	74	119.09	100.15	95.30	1.86	26.00	65.00	86.00	129.00	591.00
Individual yearly averages	98	116.65	84.89	101.06	1.62	46.42	73.33	92.66	122.50	593.42
S-PMA (µg/g creatinine)										
Spring	81	1.13	0.60	1.00	1.62	0.21	0.80	1.00	1.30	3.70
Summer	79	1.12	0.54	1.02	1.54	0.41	0.72	1.00	1.39	3.30
Autumn	76	1.53	0.93	1.33	1.67	0.49	0.97	1.29	1.84	5.80
Winter	74	1.37	0.60	1.23	1.64	0.15	1.00	1.20	1.60	3.40
Individual yearly averages	98	1.28	0.50	1.20	1.43	0.56	0.94	1.20	1.46	2.97

Table 3. Personal exposure to benzene (ln μg/m^3) by outdoor benzene concentration, cotinine, gender, age, season, province of residence, and caseness

A. Whole data-set (261 observation, 99 children)			
	β	**95% CI (β)**	**p(Z)**
Outdoor benzene(μg/m^3)	0.151	0.12; 0.19	<0.001
Gender (male vs female)	-0.052	-0.21; 0.11	0.522
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.027	-0.20; 0.25	0.814
[4-6) years	-0.147	-0.32; 0.03	0.098
Season	Reference Spring		
Summer	-0.027	-0.18; 0.12	0.717
Autumn	0.317	0.16; 0.48	<0.001
Winter	0.330	0.17; 0.49	<0.001
Residence	Reference = Turin		
Milan	-0.038	-0.28; 0.20	0.759
Florence - Rome	-0.208	-0.45; 0.03	0.091
Catania - Palermo - Cagliari	-0.086	-0.31; 0.13	0.443
Case vs control	-0.039	-0.19; 0.12	0.623
R^2 overall =0.4617 (within = 0.5364; between = 0.3603); Wald χ^2=234.0; p<0.0001			

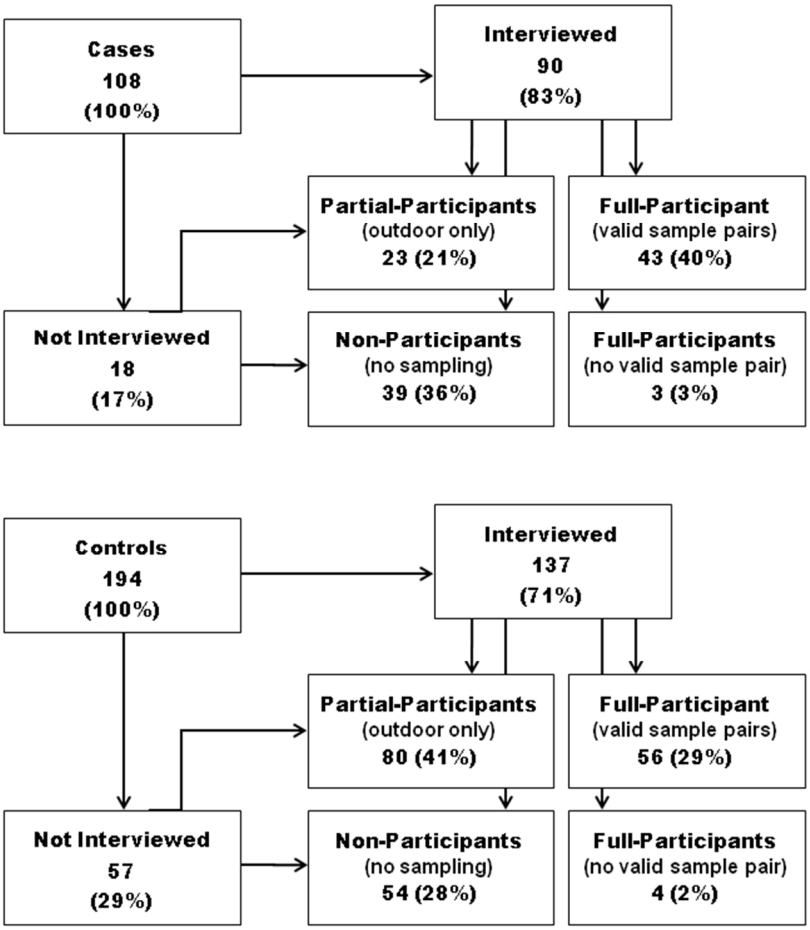
B. Restricted data-set (≥2 repeats; 175 observations, 61 children)			
	β	**SE (β)**	**p(Z)**
Outdoor benzene(μg/m^3)	0.123	0.020	<0.001
Cotinine (μg/g creatinine)	0.023	0.011	0.039
Gender (male vs female)	-0.057	0.116	0.623
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.050	0.161	0.757
[4-6) years	-0.199	0.121	0.100
Season	Reference = Spring		
Summer	-0.055	0.081	0.494
Autumn	0.382	0.087	<0.001
Winter	0.351	0.086	<0.001
Residence	Reference = Turin		
Milan	0.038	0.155	0.807
Florence - Rome	-0.323	0.195	0.099
Catania - Palermo - Cagliari	-0.00001	0.138	1.000
Case vs control	-0.073	0.107	0.498
R^2 overall =0.4858 (within = 0.5564; between = 0.3544); Wald χ^2=171.89; p<0.0001			

Table 4. Urinary excretion of S-PMA (ln µg/g creatinine) by personal benzene exposure (model 1) or outdoor benzene concentration plus urinary cotinine (model 2), controlling for gender, age, season, province of residence, and caseness

Model 1 (310 observations, 98 children)	β	**95% CI (β)**	**p(Z)**
Personal benzene exposure (µg/m^3)	0.031	0.004; 0.06	0.024
Gender (male vs female)	-0.027	-0.16; 0.11	0.695
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.395	0.22; 0.57	<0.001
[4-6) years	-0.011	-0.16; 0.14	0.890
Season	Reference Spring		
Summer	0.043	-0.09; 0.17	0.514
Autumn	0.250	0.11; 0.38	<0.001
Winter	0.156	0.01; 0.30	0.033
Residence	Reference Turin		
Milan	0.007	-0.21; 0.23	0.949
Florence - Rome	0.013	-0.18; 0.21	0.898
Catania - Palermo - Cagliari	0.068	-0.14; 0.27	0.514
Case vs control	0.053	0.647	0.415
R^2 overall =0.1894 (within = 0.1263; between = 0.2174); Wald χ^2=58.97; p <0.0001			

Model 2 (214 observations, 98 children)	β	**95% CI (β)**	**p(Z)**
Outdoor benzene concentration (µg/m^3)	0.009	-0.02; 0.04	0.605
Cotinine (µg/g creatinine)	0.014	0.001; 0.03	0.040
Gender (male vs female)	-0.012	-0.16; 0.14	0.875
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.308	0.08; 0.54	0.008
[4-6) years	0.055	-0.11; 0.22	0.516
Season	Reference Spring		
Summer	-0.040	-0.18; 0.10	0.582
Autumn	0.200	0.04; 0.36	0.012
Winter	0.082	-0.07; 0.24	0.305
Residence	Reference Turin		
Milan	-0.053	-0.28; 0.18	0.657
Florence - Rome	0.048	-0.18; 0.28	0.687
Catania - Palermo - Cagliari	0.003	-0.21; 0.22	0.974
Case vs control	0.011	-0.14; 0.16	0.882
R^2 overall =0.1158 (within = 0.1423; between = 0.0925); Wald χ^2=27.59; p = 0.0063			

Figure 1. Children eligible for inclusion and participation rates



Appendix 1 – Chemical determination: analytical conditions

Benzene concentrations in air samples

The main analytical conditions were the following: desorption at 320 °C for 10 min; overall split ratio 1:75; carrier gas nitrogen at 27 psi; column J&W PONA, 50 m, 0.2 mm id, 0.5 µm film thickness; oven 35 °C for 1 min, 6 °C/min to 110 °C, 20 °C/min to 220 °C, 2 min.

Urine analyses

Pre-treatment and chromatographic conditions used for each analyte are described below.

S-PMA. Pre-treatment of the urine sample (5 mL): calibration curve concentrations = 0, 5, 10, and 50 µg/L; acidification with HCl; centrifugation (10 minutes at 3500 rpm); purification on SPE (Isolute C18 500 mg/3 mL). Chromatographic conditions: Mobile Phase = 60% acetic acid 1% and 40% methanol; Flow = 0.3 mL/min; Column = Symmetry C18 3.0 x 150 mm, 3.5 µm (Waters); Column temperature = 29°C; Run time = 45 min; Volume injected = 21 µL; MS Method = Single Ion Recording of mass 238.0 in ESI neg; LR = 0.3 µg/L.

MA. Pre-treatment of the urine sample (2 mL): calibration curve concentrations: 0, 50, 200, 500, 1000 µg/L; centrifugation (10 minutes at 3500 rpm); purification on SPE (Isolute SAX 500 mg/3mL). Chromatographic conditions: Mobile Phase = 78 % formic acid 0.2 % and 22 % methanol; Flow = 0.3 mL/min; Column= Symmetry C18 3.0 x 150 mm, 3.5 µm (Waters); Column temperature = 30°C; Run time = 30 min; Volume injected = 21 µL. MS Method: Single Ion Recording of mass 141.0 in ESI neg; LR = 7 µg/L.

Cotinine. Pre-treatment of the urine sample (2 mL): calibration curve concentrations: 0, 10, 50, 250, 1000, 3000 µg/L; basification with Ammonium Hydroxide ACS Reagent; centrifugation (10 minutes at 3500 rpm); purification on SPE (Isolute ENV + 50 mg/3mL). Chromatographic conditions: Mobile Phase = 75 % ammonium acetate 3.7mM and 25 % methanol; Flow = 0.3 mL/min; Column = Symmetry C18 3.0 x 150 mm, 3.5 µm (Waters); Column temperature = 30°C; Run time = 33 min; Volume of sample injected = 21 µL. MS Method: Single Ion Recording of mass 177.2 in ESI pos; LR = 0.3 µg/L.

Appendix Table A. Urinary cotinine levels (ln μg/g of creatinine) by smoking habits of the parents, gender, age, season, province of residence, and caseness (295 observations from 95 children)

	β	95% CI (β)	p(Z)
Parental smoking habits	Reference Nonsmokers		
One parent smoking	0.852	0.50; 1.20	<0.001
Both parents smoking	1.685	1.22; 2.15	<0.001
Gender (male vs female)	0.028	-0.31; 0.37	0.872
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.214	-0.22; 0.65	0.338
[4-6) years	0.111	-0.27; 0.49	0.566
Season	Reference Spring		
Summer	-0.193	-0.43; 0.05	0.116
Autumn	-0.015	-0.26; 0.23	0.901
Winter	0.260	0.02; 0.50	0.035
Residence	Reference Turin		
Milan	-0.348	-0.90; 0.20	0.215
Florence - Rome	0.636	0.14; 1.13	0.011
Catania - Palermo - Cagliari	0.511	0.002; 1.02	0.049
Case vs control	0.229	-0.09; 0.55	0.164
R^2 overall =0.4213 (within = 0.0732; between = 0.5150); Wald χ^2=110.31; p<0.0001			

Appendix Table B. Participation bias factors calculated using different cut-points to dicothomize outdoor benzene concentrations

Cut-point = P50 = 3.25 μg/m^3		Exposed	Not Exposed	Bias factor
Cases	Participant	18	25	1.03
	Non Participant	11	12	
Controls	Participant	28	28	
	Non Participant	44	36	
Cut-point = P75 = 4.34 μg/m^3		**Exposed**	**Not Exposed**	**Bias factor**
Cases	Participant	4	39	0.64
	Non Participant	7	16	
Controls	Participant	14	42	
	Non Participant	26	54	
Cut-point = 5 μg/m^3		**Exposed**	**Not Exposed**	**Bias factor**
Cases	Participant	3	40	1.42
	Non Participant	4	19	
Controls	Participant	4	52	
	Non Participant	16	64	

Appendix Table C. Relationship between estimated exposures to ELF-MF (48 h TWA in the child's bedroom, ln μT) and to outdoor benzene (individual averages of repeated seasonal measurements, μg/m^3), controlling for gender, age, province of residence, caseness, and participation in the benzene pilot study (125 observations; 48 cases and 77 controls)

	β	95% CI (β)	p (t)
Outdoor benzene (μg/m^3)	0.177	0.06; 0.29	0.002
Gender (male vs female)	-0.332	-0.74; 0.08	0.112
Age (at diagnosis)	Reference [6-10] years		
[0-2) years	0.120	-0.56; 0.80	0.728
[2-4) years	0.166	-0.38; 0.72	0.550
[4-6) years	0.334	-0.29; 0.96	0.295
Residence	Reference Turin		
Milan	-0.007	-0.65; 0.64	0.984
Florence-Rome	0.135	-0.50; 0.76	0.673
Catania-Palermo-Cagliari	0.521	-0.13; 1.17	0.116
Case vs control	-0.024	-0.43; 0.38	0.908
Participant vs non participant	0.520	0.09; 0.95	0.019
F (10, 114 df) = 2.13; prob > F = 0.0271; R^2 = 0.1577			

STROBE Statement—Checklist of items that should be included in reports of ***case-control studies***

	Item No	Recommendation
Title and abstract	1★	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2★	Explain the scientific background and rationale for the investigation being reported
Objectives	3★	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4★	Present key elements of study design early in the paper
Setting	5★	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6★	(*a*) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls
		(*b*) For matched studies, give matching criteria and the number of controls per case
Variables	7★	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*★	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9★	Describe any efforts to address potential sources of bias
Study size	10★	Explain how the study size was arrived at
Quantitative variables	11★	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12★	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, explain how matching of cases and controls was addressed
		(*e*) Describe any sensitivity analyses
Results		
Participants	13*★	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*★	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
Outcome data	15*★	Report numbers in each exposure category, or summary measures of exposure
Main results	16★	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period

Other analyses	17★	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18★	Summarise key results with reference to study objectives
Limitations	19★	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20★	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21★	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22★	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

Exposure to benzene and childhood leukaemia: a pilot case-control study

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Exposure to benzene and childhood leukaemia: a pilot case-control study

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Keywords: acute lymphoblastic leukaemia, benzene, extremely low frequency magnetic fields (ELF-MF), biomarkers, children, participation bias, confounding, epidemiologic methods.

Word count: 4608

ABSTRACT

Objectives

Main purpose: to assess the feasibility of a measurement-based assessment of personal benzene exposure in case-control studies of paediatric cancer.

Additional aims: to identify the main sources of variability in personal exposure; to evaluate the performance of two benzene biomarkers; to verify the occurrence of participation bias; to check whether exposures to benzene and to 50 Hz magnetic fields were correlated, and might exert reciprocal confounding effects.

Design

Pilot case-control study of childhood leukaemia and exposure to benzene assessed by repeated seasonal weekly measurements in breathing zone air samples and outside the children's dwellings, with concurrent determinations of cotinine, *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) in urine.

Participants

Full-participation was obtained from 46 cases and 60 controls, with low dropout rates before 4 repeats (11% and 17%); additional 23 cases and 80 controls allowed collection of outdoor air samples only.

Results

The average benzene concentration in personal and outdoor air samples was 3 $\mu g/m^3$ (SD 1.45) and 2.7 $\mu g/m^3$ (SD 1.41), respectively.

Personal exposure was strongly influenced by outdoor benzene concentrations, higher in the cold seasons than in warm seasons, and not affected by gender, age, area of residence, or caseness.

Urinary excretion of S-PMA and personal benzene exposure were well correlated.

Outdoor benzene levels were lower among participant controls compared to non-participants, but did not differ between participant and non-participant cases; the direction of the bias was found to depend on the cut-point chosen to distinguish exposed and unexposed.

Exposures to benzene and ELF-MF were positively correlated.

Conclusions

Repeated individual measurements are needed to account for the seasonal variability in benzene exposure, and have the additional advantage of increasing the study power. Measurement-based assessment of benzene exposure in studies of paediatric cancer, although financially and logistically demanding, appear feasible and acceptable to children and their parents.

Article focus

- Benzene is an established causative factor for acute non lymphocytic leukaemia (AnLL), and there is limited evidence for an association between exposure to this agent and other hematologic neoplasms including acute lymphocytic leukaemia and myelodysplastic syndrome. Exposure to benzene would increase the risk of AnLL at levels of lifetime environmental exposure ≥120 ppb. While it seems unlikely that benzene is a major cause of leukaemia in the general population, children may represent a subpopulation with increased susceptibility. Available studies of benzene and childhood leukemia have provided inconsistent results, possibly due to the use of surrogate exposure proxies, and lack of analyses by leukaemia subtype. To get further insights on this topic, epidemiological studies based on objective estimates of environmental exposure to benzene have been recommended.
- Our pilot study was aimed at evaluating the logistic feasibility of an assessment of personal benzene exposure based on repeated individual measurements within a case-control study of childhood leukemia. Additional aims were: (i) to estimate the level of benzene exposure in children and assess if, and how much, exposure variability was affected by a number of putative determinants; (ii) to evaluate the performance of urinary levels of *t*-*t*-muconic acid (MA) and sulpho-phenylmercapturic acid (S-PMA) as benzene biomarkers in children; (iii) to assess the presence of participation bias (which occurs when adhesion to the study protocol is associated with both the level of exposure and the presence / absence of the disease); (iv) to determine whether exposures to benzene and to 50 Hz magnetic fields (ELF-MF) were correlated, so that they could exert reciprocal confounding effects in the analyses of their relationship with childhood leukemia.

Key messages

- Eligibility for inclusion was restricted to 108 cases and 194 matched controls, aged 2 to 12 years at the time of the survey. Full participation rates were low (cases 43%, controls 31%), but additional 21% of cases and 41% of controls accepted the outdoor monitoring. Adherence of full participants to the scheduled four seasonal repeats was very satisfactory (cases 89%, controls 83%).
- Personal exposure was strongly influenced by outdoor benzene concentrations, was higher in the cold seasons than in warm seasons, and was not affected by gender, age, area of residence, or caseness. Personal benzene exposure and urinary excretion of S-PMA (but not of

MA) were well correlated. Outdoor benzene levels were lower among participant controls compared to non-participants, but did not differ between participant and non-participant cases (a participation bias was indeed present). A positive association between exposures to benzene and ELF-MF was observed.

- Epidemiologic studies of paediatric cancer and estimates of environmental benzene exposure based on repeated seasonal measurements, although challenging, appear logistically feasible and acceptable to children and their parents.

Strengths and limitations

- To our knowledge, this is the first pilot study of childhood leukaemia and measured personal benzene exposure. Its also has the merit of having addressed a number of methodological problems besides logistic feasibility issues.
- Due to logistic reasons and resource constraints, the study size was very small. It must also be stressed that the expected greater accuracy of measurement-based exposures estimates, compared to surrogate exposure proxies, does not necessarily correspond to increased construct validity; this is especially true when measurements are used for retrospective post-diagnosis exposure assessments.

INTRODUCTION

Benzene is a ubiquitous air pollutant, that needs to be metabolized to become carcinogenic.[1- 2]

Benzene exposure and acute non lymphocytic leukaemia (AnLL) are causally related in adult humans, while there is limited evidence for an association between exposure to this agent and acute or chronic lymphocytic leukaemia, multiple myeloma, and non-Hodgkin's lymphoma.[3] Moreover, a dose-dependent association between benzene exposure and incidence of myelodysplastic syndrome has been observed among petroleum workers. [4]

Exposure to benzene would increase the risk of AnLL at levels of ≥40 ppm-years of occupational cumulative exposure, equivalent to a lifetime (76 years) environmental exposure of ≥120 ppb.[5]

Due to the established carcinogenicity of benzene, WHO has not developed any guideline value for this chemical in air, while indicating that ambient benzene concentrations of 17, 1.7 and 0.17 $\mu g/m^3$ are associated with excess lifetime risks of leukaemia of 10^{-4}, 10^{-5} and 10^{-6}, respectively.[6-7]

While it seems unlikely that benzene is a major cause of leukaemia in the general population exposed in the ppb range, children may represent a subpopulation with increased susceptibility.[1, 3]

Childhood leukaemias have distinctive features compared to leukaemias in adults. The major subtypes are acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML), accounting for 80% and 15% of cases aged 0 to 14 years in white populations respectively.[8] Both subtypes are thought to develop through a first initiating event *in utero* (e.g. the TEL-AML1 gene fusion whose prevalence in newborns has been estimated at around 1% while it is observed in 25% of ALL cases) followed by further postnatal genetic changes.[8] The "second hit" might consist of additional idiopathic chromosomal translocations, as well as of exposures to biological, chemical

or physical agents.[9] Ionizing radiation, benzene, alkylators and topoisomerase II inhibitors are among the few confirmed environmental risk factors for AML, while delayed, dysregulated responses to common infections are likely to play a major role in the conversion of pre-leukemic clones into overt ALL.[8-9]

Findings from available studies of benzene and childhood leukaemia are inconsistent, possibly due to the use of indirect estimates of exposure and lack of analyses by leukaemia subtype.[10]

To advance current understanding of benzene health effects and susceptibility, studies of paediatric cancers that include estimates of environmental exposure to benzene, rather than surrogate exposure indicators, have been recommended.[11]

Major challenges in pursuing this suggestion include the space- and time-variability of ambient benzene levels, the low exposure levels in children, and the inherent susceptibility of case-control studies (the design of choice for etiological studies of rare disease like childhood cancer) to selection and information bias.

We evaluated the logistic feasibility of an assessment of benzene exposure based on repeated seasonal weekly measurements in breathing zone air samples and outside the children's dwellings, with concurrent determinations of cotinine, *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) in urine, in a pilot investigation within an Italian case-control study on environmental risk factors for childhood leukaemia (SETIL).

Additional objectives of the pilot study were:

- to investigate the relationship between level personal exposure to benzene and putative determinants (atmospheric benzene, second-hand tobacco smoke, individual traits);
- to assess the performance of *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) as benzene biomarkers in children;

- to verify the occurrence of participation bias from differential adhesion to the benzene measurement study, and estimate the amount and direction of the distortion;
- to check whether exposures to benzene and to extremely low frequency magnetic fields (ELF-MF) were correlated, and might eventually exert reciprocal confounding effects on the relationship with childhood leukaemia.

METHODS

Study population

Incident cases of childhood leukaemia from 14 Italian regions, aged 0 to 10 years at diagnosis in 1998-2001, were eligible for enrolment in the SETIL study. Cases were ascertained through the national registry run by the Association of Paediatric Haematology and Oncology (AIEOP). Controls, matched to cases (2:1 ratio) on gender, date of birth, and region, were randomly selected from population lists. Information on several items concerning the children, their next-of-kin and dwellings, was collected by interview of parents. All interviewed families were invited to participate in a measurement study of indoor ELF-MF, while subsets of participants were asked to join two side-investigations, on exposure to gamma radiation and benzene, respectively.

Eligibility for the benzene pilot study was restricted to 108 childhood leukaemia cases from seven Italian provinces (Turin, Milan, Florence, Rome, Catania, Palermo, and Cagliari), diagnosed between July 2000 and December 2001, and 194 matched controls.

The study protocol was approved by the Piedmont Ethical Committee on 14 January 2002.

Sampling strategy and devices

Due to the high daily and seasonal variability of atmospheric benzene concentrations, the protocol called for four repeated seasonal one-week samplings of breathing zone air per child over one

year ("personal" air samples), with concurrent collection of urine samples and atmospheric air samples in proximity of the children's homes ("outdoor" air samples).

Outdoor air sampling would also be performed, with an identical strategy, near the homes of all eligible non-participants.

To study the day-to-day variability in exposure, 24-h repeated personal and indoor samples during four season-specific weeks would be collected from a subset of children and related homes.

Personal air samples were collected by passive samplers (Radiello® radial symmetry diffusive sampler) worn by the child during the day and placed at the bedside at night.

Radiello® samplers were also used to collect outdoor air samples, placed near the entrance of the dwellings (within 1 meter), at a vertical distance from the ground suitable to avoid infringements (2-2.5 m), stored in a plastic case to avoid rain or snow.

At retrieval, the adsorbing cartridges were removed from the diffusive bodies and placed into glass storage tubes. The ID code of the child, along with dates and times of sampling start and end, were recorded on self-adhesive labels stuck on the tubes. The cartridges were sent to a single laboratory (Fondazione Salvatore Maugeri, Padova) for the chemical analyses.

Daily urine samples (10 ml, from the last micturition before sleep) were collected for 7 subsequent days (70 ml per week) during each seasonal survey. The daily samples were pooled in one plastic vial, and kept in the freezer compartment of the home refrigerator until collection at the end of the week. The vials were transported to the local research centre in cool bags, and stored at –5 °C until delivery (packed in dry ice and usually in 2 weeks) to the laboratory (Fondazione Salvatore Maugeri, Pavia).

Field work began between March 2002 and January 2003, and ended in October 2003 - July 2004, depending on the local research centre.

Chemical determinations

Benzene concentrations in air sample were determined by an automated thermal desorber (ATD400, Perkin Elmer) coupled to a capillary gas-chromatography system (Autosystem XL, Perkin Elmer). The expanded uncertainty of the method, in the range 2.4 to 14.3 µg/m^3, was shown to be 18%.[12] The limits of detection and quantification, over 1 week exposure, are 0.05 µg/m^3 and 0.1 µg/m^3.

The urine analyses were performed using a high pressure liquid chromatography system (Alliance 2690, Waters) equipped with a spectrometric (SM) detector (ZQ, Waters) following a preliminary step of purification of the samples on pre-activated solid phase extraction (SPE) cartridges. The limit of detection (LOD), coefficient of variation (CV) and accuracy of the method were: LOD = 1 µg/L, CV % = (1.22)-(1.10), accuracy % = (- 2.39)-(3.36) for S-PMA; LOD = 20 µg/L, CV % = (1.33)-(1.06), accuracy % = (- 2.18)-(3.27) for MA; LOD = 1 µg/L, CV % = (1.25)-(1.09), accuracy % = (-2.29)-(3.33) for cotinine.

Further details are provided in Appendix 1.

The chemical determinations were completed by May 2005.

Statistical analyses

Measurements below the chemical-specific detection limits were assigned half such values and included in the analyses.

The relationships between personal exposure to benzene and putative determinants (as well as between urinary excretion of benzene metabolites, benzene intake, and other covariates) were assessed by generalized least squares (GLS) models for repeated measurements (STATA v. 11, xtreg procedure). The GLS model is: $y_{it} = \alpha + X_{it}B + u_{it} + e_{it}$, where i (1 to n) is the number of observations collected at time t (1 to 4) and u_{it} and e_{it} are the error components.

As concentrations of benzene and urinary analytes were log-normally distributed, we always included in the models log-transformed dependent variables.

We used the odds ratio (OR), calculated from generalized estimating equations (GEE) for repeated individual measurements (STATA v. 11, procedure xtgee), to estimate the association between benzene exposure and dichotomous variables such as case-control or participation status. The general equation of the GEE model is $g\{E(y_j)\}=x_j\beta$, where g is the link function, herein a logit function.

We calculated a participation bias factor following the method suggested by Greenland [bias factor = $(S_{1a}*S_{0b})/(S_{0a}*S_{1b})$], where S_{1a}, S_{0a}, S_{1b}, and S_{0b} denote the probabilities of selection (i.e. full participation in the benzene study) for exposed cases, unexposed cases, exposed controls, and unexposed controls.[13] When the bias factor equals 1, there is no bias, when it is above or below 1 the true OR will be biased respectively upward or downward by the magnitude of this factor.

Multiple regression models were used to analyze the relation between estimated exposures to benzene and ELF-MF.

RESULTS

Participation and sampling outcome

Out of 108 cases and 194 controls eligible for inclusion, 46 cases and 60 controls (43% and 31%) agreed to take full part in the benzene side-study (Figure 1).

In addition, the parents of 23 cases and 80 controls who refused the personal exposure assessment accepted the outdoor monitoring (partial participation = 21% and 41%).

Altogether 1467 air samples were collected. A small percentage (2%) were lost during monitoring (22 samplers stolen, 2 sampler plates broken, 3 cartridges lost), transport (8 missing labels) or

chemical analysis (2 cartridges broken on arrival at the laboratory; 1 sample lost due to equipment failure).

Benzene measurements from the day-to-day variability sub-study (19% of the total) could not be used because only four control children accepted the 24-h sampling scheme, and were replaced by the calculated weekly averages.

A further 20% of benzene measurements was removed from the data-set due to lack of compliance with the study protocol (indoor samples collected in place of the personal ones from children refusing to wear the sampler; time-or place-mismatch of personal and outdoor samples; "orphan" personal or outdoor samples; duplicate season-specific measurements; non-participants replaced with children ineligible for the benzene side-study].

For the same reasons, 107 out of 417 chemical determinations in urine (26%) were discarded.

Three cases and 5 controls were excluded from one or more analyses due to lack of complete measurement sets in all seasonal series and, although 89% and 83% of full-participant cases and controls did adhere to all four seasonal surveys, only 37% and 43% of them had four repeated analyzable observations.

Personal characteristics of the children

The families of cases participating in full to the benzene study had been interviewed on average 1.3 years (SD 0.47) after the date of diagnosis, and the control-families 1.5 years (SD 0.46) after the corresponding reference date. The delay between diagnosis and the first series of benzene measurements was 2 years (SD 0.53) for both cases and controls.

Cases and controls were comparable in terms of gender, age, and father's attained educational level (Table 1). A higher proportion of controls than cases had both parents smoking, and control-mothers were more educated than case-mothers. There were similar proportions of only children

in the case and control groups, while firstborn children were more frequent among controls than cases. Early schooling (day-care attendance) was more common in cases than in controls. At the time of the benzene survey, most children were still living in the home occupied at birth or in the house they moved into after birth but before the date of diagnosis (cases 95%; controls 91%).

Level, variability, and determinants of personal exposure to benzene

The analyses of level, variability and determinants of personal exposure to benzene were based on 43 cases (39 ALL and 4 AML) and 56 controls, with 261 valid pairs of benzene concentrations in breathing zone and outdoor air (110 from cases and 151 from controls). A large proportion of these children (35%) had a single pair of concurrent measurements, unevenly distributed by season, with a disproportionally high number of summer samples (30 out of 35, all but one from a single centre).

The distributions, overall and by season, of benzene concentrations in personal and outdoor air samples, and of cotinine, MA and S-PMA in urine are described in Table 2.

Personal exposure to benzene was log-normally distributed (Shapiro-Wilk test = 0.938, $p<0.001$), and the mean benzene level over the individual yearly averages was 3 $\mu g/m^3$ (0.92 ppb).

The distribution of benzene outdoor concentration was skewed to the left in all seasons and the yearly averages were log-normally distributed as well (Shapiro-Wilk test = 0.948, $p = 0.001$); the average yearly benzene level near the children's homes was 2.7 $\mu g/m^3$ (0.83 ppb).

Both outdoor benzene concentrations and personal exposure levels were higher in the cold seasons (autumn-winter) than in the warm ones (spring-summer).

The European limit for benzene in air (5 $\mu g/m^3$) was exceeded by 5% of the yearly average outdoor concentrations, and by 8% of the yearly average levels in breathing zone air samples. A large

proportion of autumn and winter measurements were above 5 μg/m^3 (35% and 25% outdoor; 26% and 30% of the personal exposure estimates).

Cases and controls had similar levels of personal exposure to benzene: the leukaemia OR for a unit increase (1 μg/m^3) in personal benzene exposure was 0.93 (95% CI 0.77-1.13) adjusting for gender, age at the benzene survey (2-4; 4-6; 6-12 years), cotinine in urine (μg/g creatinine), season, and province of residence (Turin; Milan; Florence - Rome; Catania - Palermo - Cagliari).

A similar lack of association was found between the odd of disease and benzene concentration outside the children's homes [OR 0.94 (95% CI 0.80-1.09)], controlling for gender, age, smoking habits of the parents at the interview (non-smokers, mother or father smoking; both parents smoking), season, and province of residence.

Further adjustment for birth order and age at first schooling had no material effect on the observed leukaemia-benzene relationship [personal exposure: OR 0.92 (95% CI 0.75-1.13); outdoor benzene: OR 0.95 (95% CI 0.81-1.13)].

As cases and controls had comparable levels of benzene exposure, we carried out the analyses illustrated in the forthcoming paragraphs on the whole data-set, although always controlling for caseness.

Urinary cotinine concentration (μg/g of creatinine) was higher in children of smoking parents compared to children of non-smokers, and children with both parents smoking excreted a larger amount of cotinine than children with one parent smoking (Appendix Table A). Cotinine levels were higher in winter than in other seasons, and higher in children from central and southern Italy (Florence, Rome, Palermo, Catania, Cagliari) than in children from northern provinces (Turin and Milan). The high between- *vs* within-subject R^2 ratio is worth noting.

Personal benzene exposure was strongly influenced by outdoor benzene concentrations (Table 3-A), and apparently not affected by gender or age; the season showed a modifying effect, with increasing levels of personal exposure during autumn and winter; the fraction of variability explained by the model was higher for the within-subject component than for the between-subject one.

Exposure to second-hand tobacco smoke (estimated by cotinine excretion or by parental smoking habits) showed a trivial influence on personal exposure to benzene. The inclusion of urinary cotinine (μg/g creatinine) in the model described in Table 3-A, slightly decreased its goodness of fit [R^2 overall = 0.46; Wald χ^2=189.49; R^2 within = 0.55; R^2 between = 0.35; β (cotinine) = 0.012; 95% CI = -0.003; 0.03)]; an alternative model, including smoking habits of the parents, did not perform any better [R^2 overall = 0.46; Wald χ^2=216.44; R^2 within = 0.52; R^2 between = 0.39; β (one parent smoking) = 0.14; 95% CI = -0.02; 0.31; β (both parents smoking) = 0.17; 95% CI = -0.06; 0.39].

Children from central Italy (Florence and Rome) tended to have lower benzene concentrations in breathing zone air samples compared to residents in other provinces, all other things being equal (Table 3-A), possibly because of residual confounding from lack of samples collected in Rome other than in summer. We tried to verify this hypothesis by restricting the analyses to children with at least two series of measurements in different seasonal periods (cold and warm). The data-set reduced to 61 subjects (25 cases and 36 controls) and 220 pairs of personal-outdoor benzene measurements. Actually, children from Florence still showed (not significantly) lower levels of personal exposure to benzene (β = - 0.27; 95% CI = -0.56; 0.03; p =0.074) compared to children from Turin. In the restricted data-set, however, independent effects of both outdoor benzene and urinary cotinine levels on personal benzene exposure were observed (Table 3-B).

Benzene intake and urinary excretion of benzene metabolites

Ninety-eight children (43 cases and 55 controls) and 310 pairs of urine and breathing zone air measurements (138 from cases and 172 from controls) were available for the analyses of the urinary excretion of benzene metabolites (MA and S-PMA) in relation to personal exposure to benzene.

Urinary concentrations of S-PMA (ln µg/g creatinine) were related to personal exposure to benzene (Table 4, Model 1). Youngest children (2-4 years at the benzene survey) excreted higher level of S-PMA compared to children aged 6-12 years, all other conditions being equal, and urinary concentration of S-PMA were higher in samples collected during the cold seasons compared to spring samples. The model, however, explained just 19% of the overall S-PMA variability. In an alternative model, including outdoor benzene concentrations and urinary cotinine in place of personal benzene exposure, we also observed an effect of the nicotine biomarker on S-PMA excretion (Table 4, Model 2).

On the contrary, neither benzene concentrations in breathing zone air samples, nor outdoor benzene concentrations or cotinine levels explained the intra- and inter-individual variability in urinary levels of MA, controlling for gender, age, season, area of residence, and caseness (data not shown).

Bias due to differential participation

Available for the analysis of participation bias were 66 cases (43 full-participant and 23 partial-participant) and 136 controls (56 and 80), with 652 measurements of outdoor benzene concentrations (135 and 175 from full-participant cases and controls; 81 and 261 from partial-participant cases and controls).

Benzene concentrations near the homes of full-participant controls were significantly lower than those in proximity of partial-participants' dwellings (OR = 0.88; 95% CI 0.80-0.97), adjusting for

gender, age, season and place of residence, while there was no difference in ambient benzene levels between participant and non-participant cases (OR = 0.95; 95% CI 0.82-1.09). As participation in the study was also associated with the case-control status, assuming a causal association between exposure and disease, a selection bias might ensue. However, as parents of more exposed controls were less willing to accept to be interviewed, an upward distortion would be expected, which is at odds with the apparent lack of association between personal benzene exposure and leukaemia risk in the current study.

To the aim of the current analysis, personal exposure to benzene was dichotomized around the median (3.25 μg/m^3), the 75th percentile (4.34 μg/m^3) or 5 μg/m^3 (the current limit for airborne benzene in Europe). The amount and direction of bias were found to depend on the cut-point chosen (Appendix Table B), whereas no bias is expected when the exposure is categorized around the median (bias factor = 1.03), and biases in the opposite directions are predicted using cut-off at p75 and at 5 μg/m^3 (0.64 and 1.42, respectively).

Relationship between exposures to benzene and ELF-MF

Children with benzene and ELF-MF measurements made at the same house qualified for inclusion in the analysis of the relationship between estimated exposures to these agents. As only 35 cases and 46 controls met such criterion when benzene concentrations in breathing zone air samples were used as exposure indicator, we performed the analysis on 48 cases and 77 controls with place-comparable pairs of average yearly outdoor benzene concentration (μg/m^3) and 48 h TWAs of ELF-MF level in the child's bedroom (ln μT).

There was a positive association between estimated exposures to ELF-MF (dependent variable) and benzene (β = 0.177; 95% CI 0.06-0.29; p = 0.002); the multivariable regression model (including gender, age, province of residence, caseness, and participation in the benzene pilot study as covariates) explained 16% of the variability in the dependent variable [F (10, 114 df) =

2.13; p> F = 0.0271]. A steeper increase in ELF-MF level per unit increase in outdoor benzene concentration (β = 0.520; 95% CI 0.09-0.95; p = 0.019) was seen among the 81 children fully participating in the benzene pilot-study compared to the 44 partial-participants (Appendix Table C).

Similar results, with a more accentuated increase in indoor magnetic induction level per unit increase in outdoor benzene concentration [β= 0.272; 95% CI = 0.09-0.45; p(t) = 0.003; R^2 = 0.19], were observed in the restricted data-set of 86 children with ≥2 weekly samplings in alternate seasons.

DISCUSSION

We have carried out a pilot case-control study of childhood leukaemia and exposure to benzene assessed by repeated individual measurements made on average two years after diagnosis. The pilot study included side-investigations aimed at evaluating the performance of two biological indicators of benzene exposure in children, at estimating amount and direction of a possible participation bias, and at assessing the relation between estimated exposures to benzene and ELF magnetic fields.

Due to the relatively low incidence of childhood cancers (10-15 for 100,000 person-years in the 0-14 year range in most industrialized countries), the case-control approach is the design of choice for analytical epidemiologic studies about potential risk factors for these diseases. Such a study design, however, is inherently prone to measurement errors stemming from the retrospective reconstruction of the exposures of interest, and to differential participation leading to control samples not representative of the study base. Therefore, findings from observational epidemiologic studies of postulated determinants for childhood malignancies are often inconsistent and always require a cautious and thoughtful interpretation.[14]

Although based on small numbers, some of the findings from the current study have a certain factual and methodological interest.

Repeated samplings of breathing and outdoor air are indeed needed to account for the seasonal variability in environmental benzene levels.[15-16]

On average, children participating in the current study appear to experience mean yearly levels of personal exposure to benzene not exceeding the European guidelines (although 8% percent of the yearly mean levels were above 5 μg/m^3).

What we *a priori* considered the main sources of benzene exposure for children (ambient benzene levels and second-hand tobacco smoke) explained no more than half of the overall variability in personal exposure, which indicates the need to identify other sources of exposure particularly relevant, perhaps, during the cold seasons. In fact, in autumn-winter compared to spring-summer, we observed higher levels of personal exposure to benzene, of urinary cotinine and of S-PMA excretion, all other things being equal. These findings might be due to the lower ventilation rates in homes and schools during the cold seasons, to winter-specific sources of indoor benzene concentrations not considered in the current survey (e.g. fireplaces or other combustion sources), and/or to the seasonal variability in daily patterns of time spent in different micro-environments (e.g. within cars or buses).[17]

Some case-control studies have suggested an association between exposure to traffic density and childhood leukaemia;[18-21] however, negative findings have also been reported.[22-25] Positive associations between incidence of ALL in children and residential proximity to petrol stations were observed in three case–control studies.[23, 26-27] An increased risk of childhood leukaemia in relation to estimated exposure to benzene was observed in a small Italian study,[28] but not in a much larger case-control study carried out in Denmark and based on a sophisticated and validated exposure modelling.[29]

To our knowledge there is no previous study of childhood leukaemia and measured personal benzene exposure. Moreover, as only children aged 0 to 10 years at diagnosis were eligible for the SETIL study, the large majority of cases included in the current investigation were pre-B ALL.

Cases and controls did not differ in terms of exposure to benzene, estimated either by benzene level in personal air samples or through outdoor benzene concentration, but the interpretation of this finding is hampered by the retrospective exposure assessment and the low statistical power of this preliminary investigation. That notwithstanding, due to the design based on repeated individual observations, the risk estimates have quite narrow confidence intervals. Thus the findings from this pilot study, in accordance with the limited evidence for an association between exposure to benzene and ALL,[3, 5] might also suggest that the levels of benzene exposure experienced by children living in Italian towns do not entail a detectable increase in the risk of ALL.

Current perspectives on the causes of childhood ALL increasingly point towards an etiologic role of altered patterns of infections and related immune stimulation during the first years of life, and one piece of supporting evidence is the consistent observation of an inverse association between ALL risk and day-care attendance.[30] Studies of childhood ALL and birth order, on the other hand, have provided inconsistent result.[31] Neither age at first schooling, nor birth order confounded the relation between childhood leukaemia and indicators of benzene exposure in the current study.

S-PMA concentration measured in repeated weekly samples of the last micturition before sleep was found to reflect personal exposure to benzene, although the available covariates explained a small fraction of the within- and between-subject variability of this benzene metabolite. This is a quite surprising result, considering that S-PMA is believed to represent less than 1% of urinary benzene metabolites for exposures to benzene at air concentrations between 0.1 and 10 ppm.[32]

Benzene exposure proved not able to explain the variability of MA urinary excretion observed in our children, consistent with findings from a previous Italian study.[33] The low statistical power of the study, the low level of benzene exposure, and the lack of adjustment for the confounding effect of dietary intake of sorbic acid (a common food additive), may explain this finding.[34]

Full-participation rates were higher among cases than controls. Notwithstanding the fairly satisfactory proportions of children with measured outdoor benzene concentrations (61% and 70% of eligible cases and controls), the degree of partial-participation was lower among non-participant cases (21%) than among non-participant controls (41%).

We observed a differential participation bias, which underscores the need to plan parallel bias analyses in any case-control study.[35] The dependence of the participation bias factor on the cut-point chosen to dichotomize the exposure variable is of methodological interest.

The positive association between the 48 h TWA of ELF-MF induction in the child's bedroom and the average yearly concentrations of outdoor benzene will need consideration in the interpretation of findings from the analyses of childhood leukaemia risk in relation to 50 Hz MF in the SETIL case-control study.

Incidental failures during sample collection, transport or chemical analysis accounted for a negligible proportion of lost air or urine samples. However, substantial percentages of chemical measurements could not be included in current analyses because of misunderstanding of the sampling protocol.

The day-to-day variability sub-study was clearly too demanding to be acceptable.

In conclusion, the current pilot study suggests that epidemiologic studies of childhood leukaemia risk and measurement-based estimates of exposure to benzene are challenging but logistically feasible (provided that the study protocol specifies every single sampling detail and nothing is

considered so obvious as to be omitted). Such an exposure assessment method could be considered by epidemiologists willing to involve in the “genome - exposome” approach to gain further insight into the relationship between benzene exposure and childhood leukaemia risk, with priority given to AML.[2, 36-38]

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COMPETING INTERESTS

None.

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Table 1. Children included in the pilot study by selected characteristics

		Cases		Controls	
		N	%	N	%
Gender	Female	25	58	30	54
	Male	18	42	26	46
Age at the survey	[2,4) years	5	12	9	16
	[4,6) years	21	49	16	29
	[6,12] years	17	40	31	55
Residence*	Turin	7	16	9	16
	Milan	8	19	13	23
	Florence	3	7	5	9
	Rome	14	33	15	27
	Catania	3	7	5	9
	Palermo	4	9	6	11
	Cagliari	4	9	3	5
Parent smoking§	None	20	47	27	48
	One	16	37	18	32
	Both	4	9	11	20
	Missing	3	7	0	-
Father's education§	No qualification	-	-	1	2
	Primary school	17	40	21	38
	High school	17	40	24	43
	University degree	6	14	10	18
	Missing	3	7	-	-
Mother's education§	No qualification	-	-	-	-
	Primary school	19	44	17	30
	High school	15	35	26	46
	University degree	9	21	13	23
	Missing	-	-	-	-
Birth order§	Only child	10	23	12	21
	First born	10	23	20	36
	Second born or higher birth order	23	53	24	43
Age at first schooling§	No schooling yet	15	35	16	29
	<3 years (crèche)	14	33	9	16
	[3,6) years (preschool)	14	33	30	54
	[6-7] years (primary school)	0	-	1	2
Home at the time of the benzene survey^	Occupied since birth	28	65	39	70
	Moved into after birth & before diagnosis	13	30	12	21
	Moved into after diagnosis & before interview	1	2	5	9
	Moved into after interview	1	2	-	-
Total		43	100	56	100

*At the time of diagnosis or the corresponding reference date for controls; §Information reported at the interview; ^The ELF magnetic fields measurements, if the parents agreed, were made at the time of the interview.

Table 2. Benzene concentration in personal and outdoor air samples, and urine level of cotinine and benzene metabolites by season and overall

	Obs (#)	Mean	SD	G-mean	G-SD	Min	Percentiles			Max
Benzene in personal air samples (µg/m^3)							**p25**	**p50**	**p75**	
Spring	57	2.51	1.89	2.10	1.75	0.60	1.50	1.82	3.11	11.12
Summer	86	2.26	1.45	1.90	1.82	0.47	1.25	1.85	3.10	8.13
Autumn	62	4.31	2.60	3.73	1.57	0.92	2.939	3.70	5.17	18.47
Winter	56	4.04	1.78	3.67	1.73	1.55	2.34	4.00	5.24	9.03
Individual yearly averages	99	3.00	1.45	2.66	1.67	0.75	2.05	2.90	3.83	9.00
Benzene in outdoor air samples (µg/m^3)										
Spring	57	2.29	1.30	1.93	1.84	0.48	1.20	1.91	3.15	5.67
Summer	86	1.94	1.20	1.65	1.75	0.39	1.12	1.58	2.28	6.92
Autumn	62	3.99	2.58	3.05	1.92	0.08	1.93	3.42	5.63	11.18
Winter	56	3.80	1.86	3.25	2.35	0.15	2.40	3.66	5.20	8.31
Individual yearly averages	99	2.70	1.41	2.33	1.78	0.27	1.59	2.37	3.63	6.92
Cotinine (µg/ g creatinine)										
Spring	78	3.92	7.04	1.91	3.26	0.05	1.00	1.94	3.50	49.0
Summer	78	3.20	5.52	1.50	3.59	0.09	0.82	1.68	3.71	41.4
Autumn	76	4.54	8.51	1.92	3.92	0.05	1.20	1.93	4.30	48.7
Winter	74	4.36	7.38	2.32	3.01	0.10	1.20	2.30	4.80	53.5
Individual yearly averages	98	3.73	5.99	2.14	2.67	0.30	1.08	2.09	3.58	41.9
MA (µg/g creatinine)										
Spring	81	104.22	69.28	87.43	1.79	17.00	60.27	82.00	126.99	349.00
Summer	79	140.40	226.73	92.30	2.16	13.33	56.54	83.00	131.76	1680.00
Autumn	76	128.24	124.04	99.57	1.94	30.21	60.16	102.48	147.21	893.04
Winter	74	119.09	100.15	95.30	1.86	26.00	65.00	86.00	129.00	591.00
Individual yearly averages	98	116.65	84.89	101.06	1.62	46.42	73.33	92.66	122.50	593.42
S-PMA (µg/g creatinine)										
Spring	81	1.13	0.60	1.00	1.62	0.21	0.80	1.00	1.30	3.70
Summer	79	1.12	0.54	1.02	1.54	0.41	0.72	1.00	1.39	3.30
Autumn	76	1.53	0.93	1.33	1.67	0.49	0.97	1.29	1.84	5.80
Winter	74	1.37	0.60	1.23	1.64	0.15	1.00	1.20	1.60	3.40
Individual yearly averages	98	1.28	0.50	1.20	1.43	0.56	0.94	1.20	1.46	2.97

Table 3. Personal exposure to benzene (ln μg/m^3) by outdoor benzene concentration, cotinine, gender, age, season, province of residence, and caseness

A. Whole data-set (261 observation, 99 children)			
	β	**95% CI (β)**	**p(Z)**
Outdoor benzene(μg/m^3)	0.151	0.12; 0.19	<0.001
Gender (male vs female)	-0.052	-0.21; 0.11	0.522
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.027	-0.20; 0.25	0.814
[4-6) years	-0.147	-0.32; 0.03	0.098
Season	Reference Spring		
Summer	-0.027	-0.18; 0.12	0.717
Autumn	0.317	0.16; 0.48	<0.001
Winter	0.330	0.17; 0.49	<0.001
Residence	Reference = Turin		
Milan	-0.038	-0.28; 0.20	0.759
Florence - Rome	-0.208	-0.45; 0.03	0.091
Catania - Palermo - Cagliari	-0.086	-0.31; 0.13	0.443
Case vs control	-0.039	-0.19; 0.12	0.623
R^2 overall =0.4617 (within = 0.5364; between = 0.3603); Wald χ^2=234.0; p<0.0001			

B. Restricted data-set (≥2 repeats; 175 observations, 61 children)			
	β	**SE (β)**	**p(Z)**
Outdoor benzene(μg/m^3)	0.123	0.020	<0.001
Cotinine (μg/g creatinine)	0.023	0.011	0.039
Gender (male vs female)	-0.057	0.116	0.623
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.050	0.161	0.757
[4-6) years	-0.199	0.121	0.100
Season	Reference = Spring		
Summer	-0.055	0.081	0.494
Autumn	0.382	0.087	<0.001
Winter	0.351	0.086	<0.001
Residence	Reference = Turin		
Milan	0.038	0.155	0.807
Florence - Rome	-0.323	0.195	0.099
Catania - Palermo - Cagliari	-0.00001	0.138	1.000
Case vs control	-0.073	0.107	0.498
R^2 overall =0.4858 (within = 0.5564; between = 0.3544); Wald χ^2=171.89; p<0.0001			

Table 4. Urinary excretion of S-PMA (ln µg/g creatinine) by personal benzene exposure (model 1) or outdoor benzene concentration plus urinary cotinine (model 2), controlling for gender, age, season, province of residence, and caseness

Model 1 (310 observations, 98 children)	β	**95% CI (β)**	**p(Z)**
Personal benzene exposure (µg/m^3)	0.031	0.004; 0.06	0.024
Gender (male vs female)	-0.027	-0.16; 0.11	0.695
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.395	0.22; 0.57	<0.001
[4-6) years	-0.011	-0.16; 0.14	0.890
Season	Reference Spring		
Summer	0.043	-0.09; 0.17	0.514
Autumn	0.250	0.11; 0.38	<0.001
Winter	0.156	0.01; 0.30	0.033
Residence	Reference Turin		
Milan	0.007	-0.21; 0.23	0.949
Florence - Rome	0.013	-0.18; 0.21	0.898
Catania - Palermo - Cagliari	0.068	-0.14; 0.27	0.514
Case vs control	0.053	0.647	0.415
R^2 overall =0.1894 (within = 0.1263; between = 0.2174); Wald χ^2=58.97; p <0.0001			

Model 2 (214 observations, 98 children)	β	**95% CI (β)**	**p(Z)**
Outdoor benzene concentration (µg/m^3)	0.009	-0.02; 0.04	0.605
Cotinine (µg/g creatinine)	0.014	0.001; 0.03	0.040
Gender (male vs female)	-0.012	-0.16; 0.14	0.875
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.308	0.08; 0.54	0.008
[4-6) years	0.055	-0.11; 0.22	0.516
Season	Reference Spring		
Summer	-0.040	-0.18; 0.10	0.582
Autumn	0.200	0.04; 0.36	0.012
Winter	0.082	-0.07; 0.24	0.305
Residence	Reference Turin		
Milan	-0.053	-0.28; 0.18	0.657
Florence - Rome	0.048	-0.18; 0.28	0.687
Catania - Palermo - Cagliari	0.003	-0.21; 0.22	0.974
Case vs control	0.011	-0.14; 0.16	0.882
R^2 overall =0.1158 (within = 0.1423; between = 0.0925); Wald χ^2=27.59; p = 0.0063			

Exposure to benzene and childhood leukaemia: a pilot case-control study

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Word count: ~~4552~~4608

ABSTRACT

Objectives

Main purpose: to assess the feasibility of a measurement-based assessment of personal benzene exposure in case-control studies of paediatric cancer.

Additional aims: to identify the main sources of variability in personal exposure; to evaluate the performance of two benzene biomarkers; to verify the occurrence of participation bias; to check whether exposures to benzene and to 50 Hz magnetic fields were correlated, and might exert reciprocal confounding effects.

Design

Pilot case-control study of childhood leukaemia and exposure to benzene assessed by repeated seasonal weekly measurements in breathing zone air samples and outside the children's dwellings, with concurrent determinations of cotinine, *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) in urine.

Participants

Full-participation was obtained from 46 cases and 60 controls, with low dropout rates before 4 repeats (11% and 17%); additional 23 cases and 80 controls allowed collection of outdoor air samples only.

Results

The average benzene concentration in personal and outdoor air samples was 3 $\mu g/m^3$ (SD 1.45) and 2.7 $\mu g/m^3$ (SD 1.41), respectively.

Personal exposure was strongly influenced by outdoor benzene concentrations, higher in the cold seasons than in warm seasons, and not affected by gender, age, area of residence, or caseness.

Urinary excretion of S-PMA and personal benzene exposure were well correlated.

Outdoor benzene levels were lower among participant controls compared to non-participants, but did not differ between participant and non-participant cases; the direction of the bias was found to depend on the cut-point chosen to distinguish exposed and unexposed.

Exposures to benzene and ELF-MF were positively correlated.

Conclusions

Repeated individual measurements are needed to account for the seasonal variability in benzene exposure, and have the additional advantage of increasing the study power. Measurement-based assessment of benzene exposure in studies of paediatric cancer, although financially and logistically demanding, appear feasible and acceptable to children and their parents.

Article focus

- Benzene is an established causative factor for acute non lymphocytic leukaemia (AnLL), and there is limited evidence ~~limited evidence~~ for an association between exposure to this agent and other hematologic neoplasms including acute lymphocytic leukaemia and myelodysplastic syndrome. Exposure to benzene would increase the risk of ~~leukaemia~~ AnLL at ~~relatively high~~ levels of lifetime environmental exposure ~~(~~≥120 ppb~~)~~. While it seems unlikely that benzene is a major cause of leukaemia in the general population, children may represent a subpopulation with increased susceptibility. Available studies of benzene and childhood leukemia have provided inconsistent results, possibly due to the use of surrogate exposure proxies, and lack of analyses by leukaemia subtype. To get further insights on this topic, epidemiological studies based on objective estimates of environmental exposure to benzene have been recommended.
- Our pilot study was aimed at evaluating the logistic feasibility of an assessment of personal benzene exposure based on repeated individual measurements within a case-control study of childhood leukemia. Additional aims were: (i) to estimate the level of benzene exposure in children and assess if, and how much, exposure variability was affected by a number of putative determinants; (ii) to evaluate the performance of urinary levels of *t*-*t*-muconic acid (MA) and sulpho-phenylmercapturic acid (S-PMA) as benzene biomarkers in children; (iii) to assess the presence of participation bias (which occurs when adhesion to the study protocol is associated with both the level of exposure and the presence / absence of the disease); (iv) to determine whether exposures to benzene and to 50 Hz magnetic fields (ELF-MF) were correlated, so that they could exert reciprocal confounding effects in the analyses of their relationship with childhood leukemia.

Key messages

- Eligibility for inclusion was restricted to 108 cases and 194 matched controls, aged 2 to 12 years at the time of the survey. Full participation rates were low (cases 43%, controls 31%), but additional 21% of cases and 41% of controls accepted the outdoor monitoring. Adherence of full participants to the scheduled four seasonal repeats was very satisfactory (cases 89%, controls 83%).
- Personal exposure was strongly influenced by outdoor benzene concentrations, was higher in the cold seasons than in warm seasons, and was not affected by gender, age, area of

residence, or caseness. Personal benzene exposure and urinary excretion of S-PMA (but not of MA) were well correlated. Outdoor benzene levels were lower among participant controls compared to non-participants, but did not differ between participant and non-participant cases (a participation bias was indeed present). A positive association between exposures to benzene and ELF-MF was observed.

- Epidemiologic studies of paediatric cancer and estimates of environmental benzene exposure based on repeated seasonal measurements, although challenging, appear logistically feasible and acceptable to children and their parents.

Strengths and limitations

- To our knowledge, this is the first pilot study of childhood leukaemia and measured personal benzene exposure. Its also has the merit of having addressed a number of methodological problems besides logistic feasibility issues.
- Due to logistic reasons and resource constraints, the study size was very small. It must also be stressed that the expected greater accuracy of measurement-based exposures estimates, compared to surrogate exposure proxies, does not necessarily correspond to increased construct validity; this is especially true when measurements are used for retrospective post-diagnosis exposure assessments.

INTRODUCTION

Benzene is a ubiquitous air pollutant, that needs to be metabolized to become carcinogenic.[1- 2]

Benzene exposure and acute non lymphocytic leukaemia (AnLL) are causally related in adult humans, while there is limited evidence for an association between exposure to this agent and acute or chronic lymphocytic leukaemia, multiple myeloma, and non-Hodgkin's lymphoma.[3] Moreover, a dose-dependent association between benzene exposure and incidence of myelodysplastic syndrome has been observed among petroleum workers. [4]

Exposure to benzene would increase the risk of AnLL ~~leukaemia~~ at levels of ≥40 ppm-years of occupational cumulative exposure, equivalent to a lifetime (76 years) environmental exposure of ≥120 ppb.[45]

Due to the established carcinogenicity of benzene, WHO has not developed any guideline value for this chemical in air, while indicating that ambient benzene concentrations of 17, 1.7 and 0.17 $\mu g/m^3$ are associated with excess lifetime risks of leukaemia of 10^{-4}, 10^{-5} and 10^{-6}, respectively.[~~5~~6-~~6~~7]

While it seems unlikely that benzene is a major cause of leukaemia in the general population exposed in the ppb range, children may represent a subpopulation with increased susceptibility ~~on intake or on key pharmacokinetic / pharmacodynamic processes~~.[1, 3]

Childhood leukaemias have distinctive features compared to leukaemias in adults. The major subtypes are acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML), accounting for 80% and 15% of cases aged 0 to 14 years in white populations respectively.[8] Both subtypes are thought to develop through a first initiating event *in utero* (e.g. the TEL-AML1 gene fusion whose prevalence in newborns has been estimated at around 1% while it is observed in 25% of ALL cases) followed by further postnatal genetic changes.[8] The "second hit" might consist of

additional idiopathic chromosomal translocations, as well as of exposures to biological, chemical or physical agents~~In precursor B-cell acute lymphoblastic leukaemia (pre-B ALL) and some cases of acute myeloid leukaemia (AML), a first initiating genetic event has been shown to occur *in utero*, at a rate of up to 1% (for TEL-AML1 translocations in pre-B ALL). Further genetic changes are required to create a malignant clone~~.[9] Ionizing radiation, benzene, alkylators and topoisomerase II inhibitors are among the few confirmed environmental risk factors for AML, while delayed, dysregulated responses to common infections are likely to play a major role in the conversion of pre-leukemic clones into overt ALL.[~~7~~8-9]

Findings from available studies of benzene and childhood leukaemia are inconsistent, possibly due to the use of indirect estimates of exposure and lack of analyses by leukaemia subtype.[~~8~~10]

To advance current understanding of benzene health effects and susceptibility, studies of paediatric cancers that include estimates of environmental exposure to benzene, rather than surrogate exposure indicators, have been recommended.[~~9~~11]

Major challenges in pursuing this suggestion include the space- and time-variability of ambient benzene levels, the low exposure levels in children, and the inherent susceptibility of case-control studies (the design of choice for etiological studies of rare disease like childhood cancer) to selection and information bias.

We evaluated the logistic feasibility of an assessment of benzene exposure based on repeated seasonal weekly measurements in breathing zone air samples and outside the children's dwellings, with concurrent determinations of cotinine, *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) in urine, in a pilot investigation within an Italian case-control study on environmental risk factors for childhood leukaemia (SETIL).

Additional objectives of the pilot study were:

- to investigate the relationship between level personal exposure to benzene and putative determinants (atmospheric benzene, second-hand tobacco smoke, individual traits);
- to assess the performance of *t-t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) as benzene biomarkers in children;
- to verify the occurrence of participation bias from differential adhesion to the benzene measurement study, and estimate the amount and direction of the distortion;
- to check whether exposures to benzene and to extremely low frequency magnetic fields (ELF-MF) were correlated, and might eventually exert reciprocal confounding effects on the relationship with childhood leukaemia.

METHODS

Study population

Incident cases of childhood leukaemia from 14 Italian regions, aged 0 to 10 years at diagnosis in 1998-2001, were eligible for enrolment in the SETIL study. Cases were ascertained through the national registry run by the Association of Paediatric Haematology and Oncology (AIEOP). Controls, matched to cases (2:1 ratio) on gender, date of birth, and region, were randomly selected from population lists. Information on several items concerning the children, their next-of-kin and dwellings, was collected by interview of parents. All interviewed families were invited to participate in a measurement study of indoor ELF-MF, while subsets of participants were asked to join two side-investigations, on exposure to gamma radiation and benzene, respectively.

Eligibility for the benzene pilot study was restricted to 108 childhood leukaemia cases from seven Italian provinces (Turin, Milan, Florence, Rome, Catania, Palermo, and Cagliari), diagnosed between July 2000 and December 2001, and 194 matched controls.

The study protocol was approved by the Piedmont Ethical Committee on 14 January 2002.

Sampling strategy and devices

Due to the high daily and seasonal variability of atmospheric benzene concentrations, the protocol called for four repeated seasonal one-week samplings of breathing zone air per child over one year ("personal" air samples), with concurrent collection of urine samples and atmospheric air samples in proximity of the children's homes ("outdoor" air samples).

Outdoor air sampling would also be performed, with an identical strategy, near the homes of all eligible non-participants.

To study the day-to-day variability in exposure, 24-h repeated personal and indoor samples during four season-specific weeks would be collected from a subset of children and related homes.

Personal air samples were collected by passive samplers (Radiello® radial symmetry diffusive sampler) worn by the child during the day and placed at the bedside at night.

Radiello® samplers were also used to collect outdoor air samples, placed near the entrance of the dwellings (within 1 meter), at a vertical distance from the ground suitable to avoid infringements (2-2.5 m), stored in a plastic case to avoid rain or snow.

At retrieval, the adsorbing cartridges were removed from the diffusive bodies and placed into glass storage tubes. The ID code of the child, along with dates and times of sampling start and end, were recorded on self-adhesive labels stuck on the tubes. The cartridges were sent to a single laboratory (Fondazione Salvatore Maugeri, Padova) for the chemical analyses.

Daily urine samples (10 ml, from the last micturition before sleep) were collected for 7 subsequent days (70 ml per week) during each seasonal survey. The daily samples were pooled in one plastic vial, and kept in the freezer compartment of the home refrigerator until collection at the end of the week. The vials were transported to the local research centre in cool bags, and stored at –5 °C

until delivery (packed in dry ice and usually in 2 weeks) to the laboratory (Fondazione Salvatore Maugeri, Pavia).

Field work began between March 2002 and January 2003, and ended in October 2003 - July 2004, depending on the local research centre.

Chemical determinations

Benzene concentrations in air sample were determined by an automated thermal desorber (ATD400, Perkin Elmer) coupled to a capillary gas-chromatography system (Autosystem XL, Perkin Elmer). The expanded uncertainty of the method, in the range 2.4 to 14.3 µg/m^3, was shown to be 18%.[~~10~~12] The limits of detection and quantification, over 1 week exposure, are 0.05 µg/m^3 and 0.1 µg/m^3.

The urine analyses were performed using a high pressure liquid chromatography system (Alliance 2690, Waters) equipped with a spectrometric (SM) detector (ZQ, Waters) following a preliminary step of purification of the samples on pre-activated solid phase extraction (SPE) cartridges. The limit of detection (LOD), coefficient of variation (CV) and accuracy of the method were: LOD = 1 µg/L, CV % = (1.22)-(1.10), accuracy % = (- 2.39)-(3.36) for S-PMA; LOD = 20 µg/L, CV % = (1.33)-(1.06), accuracy % = (- 2.18)-(3.27) for MA; LOD = 1 µg/L, CV % = (1.25)-(1.09), accuracy % = (-2.29)-(3.33) for cotinine.

Further details are provided in Appendix 1.

The chemical determinations were completed by May 2005.

Statistical analyses

Measurements below the chemical-specific detection limits were assigned half such values and included in the analyses.

The relationships between personal exposure to benzene and putative determinants (as well as between urinary excretion of benzene metabolites, benzene intake, and other covariates) were assessed by generalized least squares (GLS) models for repeated measurements (STATA v. 11, xtreg procedure). The GLS model is: $y_{it} = \alpha + X_{it}B + u_{it} + e_{it}$, where i (1 to n) is the number of observations collected at time t (1 to 4) and u_{it} and e_{it} are the error components.

As concentrations of benzene and urinary analytes were log-normally distributed, we always included in the models log-transformed dependent variables.

We used the odds ratio (OR), calculated from generalized estimating equations (GEE) for repeated individual measurements (STATA v. 11, procedure xtgee), to estimate the association between benzene exposure and dichotomous variables such as case-control or participation status. The general equation of the GEE model is $g\{E(y_j)\}=x_j\beta$, where g is the link function, herein a logit function.

We calculated a participation bias factor following the method suggested by Greenland [bias factor = $(S_{1a}*S_{0b})/(S_{0a}*S_{1b})$], where S_{1a}, S_{0a}, S_{1b}, and S_{0b} denote the probabilities of selection (i.e. full participation in the benzene study) for exposed cases, unexposed cases, exposed controls, and unexposed controls.[~~11~~13] When the bias factor equals 1, there is no bias, when it is above or below 1 the true OR will be biased respectively upward or downward by the magnitude of this factor.

Multiple regression models were used to analyze the relation between estimated exposures to benzene and ELF-MF.

RESULTS

Participation and sampling outcome

Out of 108 cases and 194 controls eligible for inclusion, 46 cases and 60 controls (43% and 31%) agreed to take full part in the benzene side-study (Figure 1).

In addition, the parents of 23 cases and 80 controls who refused the personal exposure assessment accepted the outdoor monitoring (partial participation = 21% and 41%).

Altogether 1467 air samples were collected. A small percentage (2%) were lost during monitoring (22 samplers stolen, 2 sampler plates broken, 3 cartridges lost), transport (8 missing labels) or chemical analysis (2 cartridges broken on arrival at the laboratory; 1 sample lost due to equipment failure).

Benzene measurements from the day-to-day variability sub-study (19% of the total) could not be used because only four control children accepted the 24-h sampling scheme, and were replaced by the calculated weekly averages.

A further 20% of benzene measurements was removed from the data-set due to lack of compliance with the study protocol (indoor samples collected in place of the personal ones from children refusing to wear the sampler; time-or place-mismatch of personal and outdoor samples; "orphan" personal or outdoor samples; duplicate season-specific measurements; non-participants replaced with children ineligible for the benzene side-study].

For the same reasons, 107 out of 417 chemical determinations in urine (26%) were discarded.

Three cases and 5 controls were excluded from one or more analyses due to lack of complete measurement sets in all seasonal series and, although 89% and 83% of full-participant cases and controls did adhere to all four seasonal surveys, only 37% and 43% of them had four repeated analyzable observations.

Personal characteristics of the children

The families of cases participating in full to the benzene study had been interviewed on average 1.3 years (SD 0.47) after the date of diagnosis, and the control-families 1.5 years (SD 0.46) after the corresponding reference date. The delay between diagnosis and the first series of benzene measurements was 2 years (SD 0.53) for both cases and controls.

Cases and controls were comparable in terms of gender, age, and father's attained educational level (Table 1). A higher proportion of controls than cases had both parents smoking, and control-mothers were more educated than case-mothers. There were similar proportions of only children in the case and control groups, while firstborn children were more frequent among controls than cases. Early schooling (day-care attendance ~~of crèche~~) was more common in cases than in controls. At the time of the benzene survey, most children were still living in the home occupied at birth or in the house they moved into after birth but before the date of diagnosis (cases 95%; controls 91%).

Level, variability, and determinants of personal exposure to benzene

The analyses of level, variability and determinants of personal exposure to benzene were based on 43 cases (39 ALL and 4 AML) and 56 controls, with 261 valid pairs of benzene concentrations in breathing zone and outdoor air (110 from cases and 151 from controls). A large proportion of these children (35%) had a single pair of concurrent measurements, unevenly distributed by season, with a disproportionally high number of summer samples (30 out of 35, all but one from a single centre).

The distributions, overall and by season, of benzene concentrations in personal and outdoor air samples, and of cotinine, MA and S-PMA in urine are described in Table 2.

Personal exposure to benzene was log-normally distributed (Shapiro-Wilk test = 0.938, $p<0.001$), and the mean benzene level over the individual yearly averages was 3 $\mu g/m^3$ (0.92 ppb).

The distribution of benzene outdoor concentration was skewed to the left in all seasons and the yearly averages were log-normally distributed as well (Shapiro-Wilk test = 0.948, p = 0.001); the average yearly benzene level near the children's homes was 2.7 μg/m^3 (0.83 ppb).

Both outdoor benzene concentrations and personal exposure levels were higher in the cold seasons (autumn-winter) than in the warm ones (spring-summer).

The European limit for benzene in air (5 μg/m^3) was exceeded by 5% of the yearly average outdoor concentrations, and by 8% of the yearly average levels in breathing zone air samples. A large proportion of autumn and winter measurements were above 5 μg/m^3 (35% and 25% outdoor; 26% and 30% of the personal exposure estimates).

Cases and controls had similar levels of personal exposure to benzene: the leukaemia OR for a unit increase (1 μg/m^3) in personal benzene exposure was 0.93 (95% CI 0.77-1.13) adjusting for gender, age at the benzene survey (2-4; 4-6; 6-12 years), cotinine in urine (μg/g creatinine), season, and province of residence (Turin; Milan; Florence - Rome; Catania - Palermo - Cagliari).

A similar lack of association was found between the odd of disease and benzene concentration outside the children's homes [OR 0.94 (95% CI 0.80-1.09)], controlling for gender, age, smoking habits of the parents at the interview (non-smokers, mother or father smoking; both parents smoking), season, and province of residence.

Further adjustment for birth order and age at first schooling had no material effect on the observed leukaemia-benzene relationship [personal exposure: OR 0.92 (95% CI 0.75-1.13); outdoor benzene: OR 0.95 (95% CI 0.81-1.13)].

As cases and controls had comparable levels of benzene exposure, we carried out the analyses illustrated in the forthcoming paragraphs on the whole data-set, although always controlling for caseness.

Urinary cotinine concentration (μg/g of creatinine) was higher in children of smoking parents compared to children of non-smokers, and children with both parents smoking excreted a larger amount of cotinine than children with one parent smoking (Appendix Table A). Cotinine levels were higher in winter than in other seasons, and higher in children from central and southern Italy (Florence, Rome, Palermo, Catania, Cagliari) than in children from northern provinces (Turin and Milan). The high between- *vs* within-subject R^2 ratio is worth noting.

Personal benzene exposure was strongly influenced by outdoor benzene concentrations (Table 3-A), and apparently not affected by gender or age; the season showed a modifying effect, with increasing levels of personal exposure during autumn and winter; the fraction of variability explained by the model was higher for the within-subject component than for the between-subject one.

Exposure to second-hand tobacco smoke (estimated by cotinine excretion or by parental smoking habits) showed a trivial influence on personal exposure to benzene. The inclusion of urinary cotinine (μg/g creatinine) in the model described in Table 3-A, slightly decreased its goodness of fit [R^2 overall = 0.46; Wald χ^2=189.49; R^2 within = 0.55; R^2 between = 0.35; β (cotinine) = 0.012; 95% CI = -0.003; 0.03)]; an alternative model, including smoking habits of the parents, did not perform any better [R^2 overall = 0.46; Wald χ^2=216.44; R^2 within = 0.52; R^2 between = 0.39; β (one parent smoking) = 0.14; 95% CI = -0.02; 0.31; β (both parents smoking) = 0.17; 95% CI = -0.06; 0.39].

Children from central Italy (Florence and Rome) tended to have lower benzene concentrations in breathing zone air samples compared to residents in other provinces, all other things being equal (Table 3-A), possibly because of residual confounding from lack of samples collected in Rome other than in summer. We tried to verify this hypothesis by restricting the analyses to children with at least two series of measurements in different seasonal periods (cold and warm). The data-set reduced to 61 subjects (25 cases and 36 controls) and 220 pairs of personal-outdoor benzene

measurements. Actually, children from Florence still showed (not significantly) lower levels of personal exposure to benzene (β = - 0.27; 95% CI = -0.56; 0.03; p =0.074) compared to children from Turin. In the restricted data-set, however, independent effects of both outdoor benzene and urinary cotinine levels on personal benzene exposure were observed (Table 3-B).

Benzene intake and urinary excretion of benzene metabolites

Ninety-eight children (43 cases and 55 controls) and 310 pairs of urine and breathing zone air measurements (138 from cases and 172 from controls) were available for the analyses of the urinary excretion of benzene metabolites (MA and S-PMA) in relation to personal exposure to benzene.

Urinary concentrations of S-PMA (ln µg/g creatinine) were related to personal exposure to benzene (Table 4, Model 1). Youngest children (2-4 years at the benzene survey) excreted higher level of S-PMA compared to children aged 6-12 years, all other conditions being equal, and urinary concentration of S-PMA were higher in samples collected during the cold seasons compared to spring samples. The model, however, explained just 19% of the overall S-PMA variability. In an alternative model, including outdoor benzene concentrations and urinary cotinine in place of personal benzene exposure, we also observed an effect of the nicotine biomarker on S-PMA excretion (Table 4, Model 2).

On the contrary, neither benzene concentrations in breathing zone air samples, nor outdoor benzene concentrations or cotinine levels explained the intra- and inter-individual variability in urinary levels of MA, controlling for gender, age, season, area of residence, and caseness (data not shown).

Bias due to differential participation

Available for the analysis of participation bias were 66 cases (43 full-participant and 23 partial-participant) and 136 controls (56 and 80), with 652 measurements of outdoor benzene concentrations (135 and 175 from full-participant cases and controls; 81 and 261 from partial-participant cases and controls).

Benzene concentrations near the homes of full-participant controls were significantly lower than those in proximity of partial-participants' dwellings (OR = 0.88; 95% CI 0.80-0.97), adjusting for gender, age, season and place of residence, while there was no difference in ambient benzene levels between participant and non-participant cases (OR = 0.95; 95% CI 0.82-1.09). As participation in the study was also associated with the case-control status, assuming a causal association between exposure and disease, a selection bias might ensue. However, as parents of more exposed controls were less willing to accept to be interviewed, an upward distortion would be expected, which is at odds with the apparent lack of association between personal benzene exposure and leukaemia risk in the current study.

To the aim of the current analysis, personal exposure to benzene was dichotomized around the median (3.25 $\mu g/m^3$), the 75th percentile (4.34 $\mu g/m^3$) or 5 $\mu g/m^3$ (the current limit for airborne benzene in Europe). The amount and direction of bias were found to depend on the cut-point chosen (Appendix Table B), whereas no bias is expected when the exposure is categorized around the median (bias factor = 1.03), and biases in the opposite directions are predicted using cut-off at p75 and at 5 $\mu g/m^3$ (0.64 and 1.42, respectively).

Relationship between exposures to benzene and ELF-MF

Children with benzene and ELF-MF measurements made at the same house qualified for inclusion in the analysis of the relationship between estimated exposures to these agents. As only 35 cases and 46 controls met such criterion when benzene concentrations in breathing zone air samples were used as exposure indicator, we performed the analysis on 48 cases and 77 controls with

place-comparable pairs of average yearly outdoor benzene concentration (μg/m^3) and 48 h TWAs of ELF-MF level in the child's bedroom (ln μT).

There was a positive association between estimated exposures to ELF-MF (dependent variable) and benzene (β = 0.177; 95% CI 0.06-0.29; p = 0.002); the multivariable regression model (including gender, age, province of residence, caseness, and participation in the benzene pilot study as covariates) explained 16% of the variability in the dependent variable [F (10, 114 df) = 2.13; p> F = 0.0271]. A steeper increase in ELF-MF level per unit increase in outdoor benzene concentration (β = 0.520; 95% CI 0.09-0.95; p = 0.019) was seen among the 81 children fully participating in the benzene pilot-study compared to the 44 partial-participants (Appendix Table C).

Similar results, with a more accentuated increase in indoor magnetic induction level per unit increase in outdoor benzene concentration [β= 0.272; 95% CI = 0.09-0.45; p(t) = 0.003; R^2 = 0.19], were observed in the restricted data-set of 86 children with ≥2 weekly samplings in alternate seasons.

DISCUSSION

We have carried out a pilot case-control study of childhood leukaemia and exposure to benzene assessed by repeated individual measurements made on average two years after diagnosis. The pilot study included side-investigations aimed at evaluating the performance of two biological indicators of benzene exposure in children, at estimating amount and direction of a possible participation bias, and at assessing the relation between estimated exposures to benzene and ELF magnetic fields.

Due to the relatively low incidence of childhood cancers (10-15 for 100,000 person-years in the 0-14 year range in most industrialized countries), the case-control approach is the design of choice

for analytical epidemiologic studies about potential risk factors for these diseases. Such a study design, however, is inherently prone to measurement errors stemming from the retrospective reconstruction of the exposures of interest, and to differential participation leading to control samples not representative of the study base. Therefore, findings from observational epidemiologic studies of postulated determinants for childhood malignancies are often inconsistent and always require a cautious and thoughtful interpretation.[~~12~~14]

Although based on small numbers, some of the findings from the current study have a certain factual and methodological interest.

Repeated samplings of breathing and outdoor air are indeed needed to account for the seasonal variability in environmental benzene levels.[~~13~~15-~~14~~16]

On average, children participating in the current study appear to experience mean yearly levels of personal exposure to benzene not exceeding the European guidelines (although 8% percent of the yearly mean levels were above 5 $\mu g/m^3$).

What we *a priori* considered the main sources of benzene exposure for children (ambient benzene levels and second-hand tobacco smoke) explained no more than half of the overall variability in personal exposure, which indicates the need to identify other sources of exposure particularly relevant, perhaps, during the cold seasons. In fact, in autumn-winter compared to spring-summer, we observed higher levels of personal exposure to benzene, of urinary cotinine and of S-PMA excretion, all other things being equal. These findings might be due to the lower ventilation rates in homes and schools during the cold seasons, to winter-specific sources of indoor benzene concentrations not considered in the current survey (e.g. fireplaces or other combustion sources), and/or to the seasonal variability in daily patterns of time spent in different micro-environments (e.g. within cars or buses).[~~15~~17]

Some case-control studies have suggested an association between exposure to traffic density and childhood leukaemia;[~~16~~18-~~19~~21] however, negative findings have also been reported.[~~20~~22-~~23~~25] Positive associations between incidence of ALL in children and residential proximity to petrol stations were observed in three case–control studies.[~~21~~23, ~~24~~26-~~25~~27] An increased risk of childhood leukaemia in relation to estimated exposure to benzene was observed in a small Italian study,[~~26~~28] but not in a much larger case-control study carried out in Denmark and based on a sophisticated and validated exposure modelling.[~~27~~29]

To our knowledge there is no previous study of childhood leukaemia and measured personal benzene exposure. Moreover, as only children aged 0 to 10 years at diagnosis were eligible for the SETIL study, the large majority of cases included in the current investigation were pre-B ALL.

Cases and controls did not differ in terms of exposure to benzene, estimated either by benzene level in personal air samples or through outdoor benzene concentration, but the interpretation of this finding is hampered by the retrospective exposure assessment and the low statistical power of this preliminary investigation. That notwithstanding, due to the design based on repeated individual observations, the risk estimates have quite narrow confidence intervals. Thus the findings from this pilot study, in accordance with the limited evidence for an association between exposure to benzene and ALL,[3~~-~~, 45] might also suggest that the levels of benzene exposure experienced by children living in Italian towns do not entail a detectable increase in the risk of ALL.

Current perspectives on the causes of childhood ~~leukaemia~~ ALL increasingly point towards an etiologic role of altered patterns of infections and related immune stimulation during the first years of life, and one piece of supporting evidence is the consistent observation of an inverse association between ALL risk and day-care attendance.[~~28~~30] Studies of childhood ALL and birth order, on the other hand, have provided inconsistent result.[~~29~~31] Neither age at first schooling,

nor birth order confounded the relation between childhood leukaemia and indicators of benzene exposure in the current study.

S-PMA concentration measured in repeated weekly samples of the last micturition before sleep was found to reflect personal exposure to benzene, although the available covariates explained a small fraction of the within- and between-subject variability of this benzene metabolite. This is a quite surprising result, considering that S-PMA is believed to represent less than 1% of urinary benzene metabolites for exposures to benzene at air concentrations between 0.1 and 10 ppm.[~~30~~32]

Benzene exposure proved not able to explain the variability of MA urinary excretion observed in our children, consistent with findings from a previous Italian study.[~~31~~33] The low statistical power of the study, the low level of benzene exposure, and the lack of adjustment for the confounding effect of dietary intake of sorbic acid (a common food additive), may explain this finding.[~~32~~34]

Full-participation rates were higher among cases than controls. Notwithstanding the fairly satisfactory proportions of children with measured outdoor benzene concentrations (61% and 70% of eligible cases and controls), the degree of partial-participation was lower among non-participant cases (21%) than among non-participant controls (41%).

We observed a differential participation bias, which underscores the need to plan parallel bias analyses in any case-control study.[~~33~~35] The dependence of the participation bias factor on the cut-point chosen to dichotomize the exposure variable is of methodological interest.

The positive association between the 48 h TWA of ELF-MF induction in the child's bedroom and the average yearly concentrations of outdoor benzene will need consideration in the interpretation of findings from the analyses of childhood leukaemia risk in relation to 50 Hz MF in the SETIL case-control study.

Incidental failures during sample collection, transport or chemical analysis accounted for a negligible proportion of lost air or urine samples. However, substantial percentages of chemical measurements could not be included in current analyses because of misunderstanding of the sampling protocol.

The day-to-day variability sub-study was clearly too demanding to be acceptable.

In conclusion, the current pilot study suggests that epidemiologic studies of childhood leukaemia risk and measurement-based estimates of exposure to benzene are challenging but logistically feasible (provided that the study protocol specifies every single sampling detail and nothing is considered so obvious as to be omitted). Such an exposure assessment method could be considered by epidemiologists willing to involve in the "genome - exposome" approach to gain further insight into the relationship between benzene exposure and childhood leukaemia risk, with priority given to AML.[42, 3436-38]

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COMPETING INTERESTS

None.

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Table 1. Children included in the pilot study by selected characteristics

		Cases		Controls	
		N	%	N	%
Gender	Female	25	58	30	54
	Male	18	42	26	46
Age at the survey	[2,4) years	5	12	9	16
	[4,6) years	21	49	16	29
	[6,12] years	17	40	31	55
Residence*	Turin	7	16	9	16
	Milan	8	19	13	23
	Florence	3	7	5	9
	Rome	14	33	15	27
	Catania	3	7	5	9
	Palermo	4	9	6	11
	Cagliari	4	9	3	5
Parent smoking§	None	20	47	27	48
	One	16	37	18	32
	Both	4	9	11	20
	Missing	3	7	0	-
Father's education§	No qualification	-	-	1	2
	Primary school	17	40	21	38
	High school	17	40	24	43
	University degree	6	14	10	18
	Missing	3	7	-	-
Mother's education§	No qualification	-	-	-	-
	Primary school	19	44	17	30
	High school	15	35	26	46
	University degree	9	21	13	23
	Missing	-	-	-	-
Birth order§	Only child	10	23	12	21
	First born	10	23	20	36
	Second born or higher birth order	23	53	24	43
Age at first schooling§	No schooling yet	15	35	16	29
	<3 years (crèche)	14	33	9	16
	[3,6) years (preschool)	14	33	30	54
	[6-7] years (primary school)	0	-	1	2
Home at the time of the benzene survey^	Occupied since birth	28	65	39	70
	Moved into after birth & before diagnosis	13	30	12	21
	Moved into after diagnosis & before interview	1	2	5	9
	Moved into after interview	1	2	-	-
Total		43	100	56	100

*At the time of diagnosis or the corresponding reference date for controls; §Information reported at the interview; ^The ELF magnetic fields measurements, if the parents agreed, were made at the time of the interview.

Table 2. Benzene concentration in personal and outdoor air samples, and urine level of cotinine and benzene metabolites by season and overall

	Obs (#)	Mean	SD	G-mean	G-SD	Min	Percentiles			Max
Benzene in personal air samples (μg/m^3)							**p25**	**p50**	**p75**	
Spring	57	2.51	1.89	2.10	1.75	0.60	1.50	1.82	3.11	11.12
Summer	86	2.26	1.45	1.90	1.82	0.47	1.25	1.85	3.10	8.13
Autumn	62	4.31	2.60	3.73	1.57	0.92	2.939	3.70	5.17	18.47
Winter	56	4.04	1.78	3.67	1.73	1.55	2.34	4.00	5.24	9.03
Individual yearly averages	99	3.00	1.45	2.66	1.67	0.75	2.05	2.90	3.83	9.00
Benzene in outdoor air samples (μg/m^3)										
Spring	57	2.29	1.30	1.93	1.84	0.48	1.20	1.91	3.15	5.67
Summer	86	1.94	1.20	1.65	1.75	0.39	1.12	1.58	2.28	6.92
Autumn	62	3.99	2.58	3.05	1.92	0.08	1.93	3.42	5.63	11.18
Winter	56	3.80	1.86	3.25	2.35	0.15	2.40	3.66	5.20	8.31
Individual yearly averages	99	2.70	1.41	2.33	1.78	0.27	1.59	2.37	3.63	6.92
Cotinine (μg/ g creatinine)										
Spring	78	3.92	7.04	1.91	3.26	0.05	1.00	1.94	3.50	49.0
Summer	78	3.20	5.52	1.50	3.59	0.09	0.82	1.68	3.71	41.4
Autumn	76	4.54	8.51	1.92	3.92	0.05	1.20	1.93	4.30	48.7
Winter	74	4.36	7.38	2.32	3.01	0.10	1.20	2.30	4.80	53.5
Individual yearly averages	98	3.73	5.99	2.14	2.67	0.30	1.08	2.09	3.58	41.9
MA (μg/g creatinine)										
Spring	81	104.22	69.28	87.43	1.79	17.00	60.27	82.00	126.99	349.00
Summer	79	140.40	226.73	92.30	2.16	13.33	56.54	83.00	131.76	1680.00
Autumn	76	128.24	124.04	99.57	1.94	30.21	60.16	102.48	147.21	893.04
Winter	74	119.09	100.15	95.30	1.86	26.00	65.00	86.00	129.00	591.00
Individual yearly averages	98	116.65	84.89	101.06	1.62	46.42	73.33	92.66	122.50	593.42
S-PMA (μg/g creatinine)										
Spring	81	1.13	0.60	1.00	1.62	0.21	0.80	1.00	1.30	3.70
Summer	79	1.12	0.54	1.02	1.54	0.41	0.72	1.00	1.39	3.30
Autumn	76	1.53	0.93	1.33	1.67	0.49	0.97	1.29	1.84	5.80
Winter	74	1.37	0.60	1.23	1.64	0.15	1.00	1.20	1.60	3.40
Individual yearly averages	98	1.28	0.50	1.20	1.43	0.56	0.94	1.20	1.46	2.97

Table 3. Personal exposure to benzene (ln μg/m^3) by outdoor benzene concentration, cotinine, gender, age, season, province of residence, and caseness

A. Whole data-set (261 observation, 99 children)			
	β	**95% CI (β)**	**p(Z)**
Outdoor benzene(μg/m^3)	0.151	0.12; 0.19	<0.001
Gender (male vs female)	-0.052	-0.21; 0.11	0.522
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.027	-0.20; 0.25	0.814
[4-6) years	-0.147	-0.32; 0.03	0.098
Season	Reference Spring		
Summer	-0.027	-0.18; 0.12	0.717
Autumn	0.317	0.16; 0.48	<0.001
Winter	0.330	0.17; 0.49	<0.001
Residence	Reference = Turin		
Milan	-0.038	-0.28; 0.20	0.759
Florence - Rome	-0.208	-0.45; 0.03	0.091
Catania - Palermo - Cagliari	-0.086	-0.31; 0.13	0.443
Case vs control	-0.039	-0.19; 0.12	0.623
R^2 overall =0.4617 (within = 0.5364; between = 0.3603); Wald χ^2=234.0; p<0.0001			

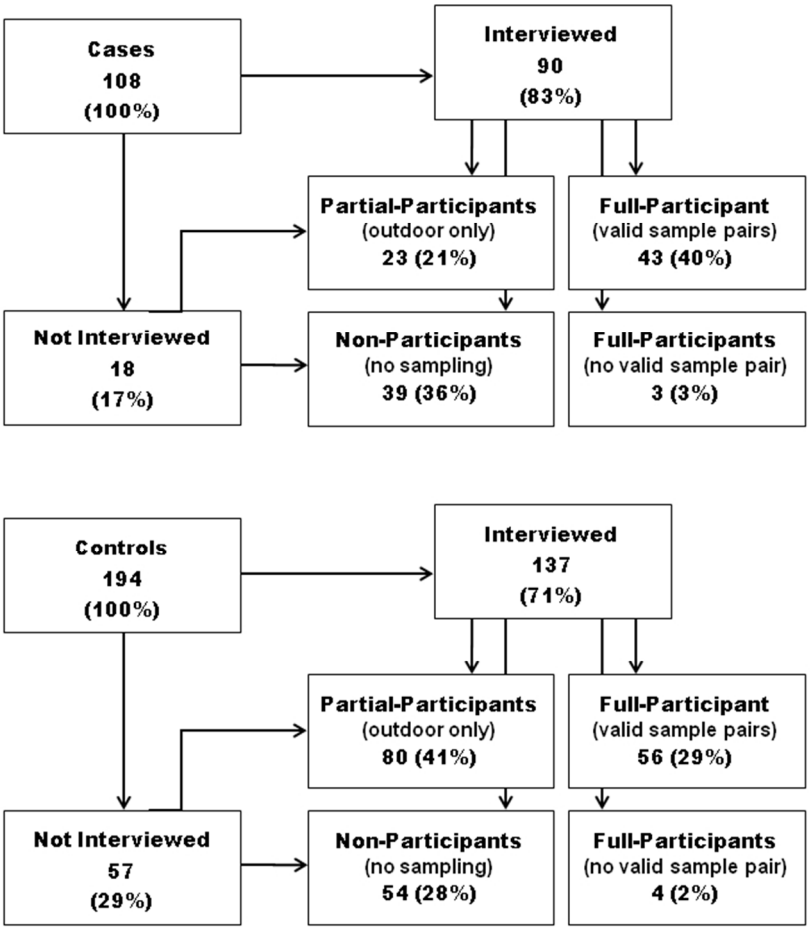
B. Restricted data-set (≥2 repeats; 175 observations, 61 children)			
	β	**SE (β)**	**p(Z)**
Outdoor benzene(μg/m^3)	0.123	0.020	<0.001
Cotinine (μg/g creatinine)	0.023	0.011	0.039
Gender (male vs female)	-0.057	0.116	0.623
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.050	0.161	0.757
[4-6) years	-0.199	0.121	0.100
Season	Reference = Spring		
Summer	-0.055	0.081	0.494
Autumn	0.382	0.087	<0.001
Winter	0.351	0.086	<0.001
Residence	Reference = Turin		
Milan	0.038	0.155	0.807
Florence - Rome	-0.323	0.195	0.099
Catania - Palermo - Cagliari	-0.00001	0.138	1.000
Case vs control	-0.073	0.107	0.498
R^2 overall =0.4858 (within = 0.5564; between = 0.3544); Wald χ^2=171.89; p<0.0001			

Table 4. Urinary excretion of S-PMA (ln µg/g creatinine) by personal benzene exposure (model 1) or outdoor benzene concentration plus urinary cotinine (model 2), controlling for gender, age, season, province of residence, and caseness

Model 1 (310 observations, 98 children)	**β**	**95% CI (β)**	**p(Z)**
Personal benzene exposure (µg/m^3)	0.031	0.004; 0.06	0.024
Gender (male vs female)	-0.027	-0.16; 0.11	0.695
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.395	0.22; 0.57	<0.001
[4-6) years	-0.011	-0.16; 0.14	0.890
Season	Reference Spring		
Summer	0.043	-0.09; 0.17	0.514
Autumn	0.250	0.11; 0.38	<0.001
Winter	0.156	0.01; 0.30	0.033
Residence	Reference Turin		
Milan	0.007	-0.21; 0.23	0.949
Florence - Rome	0.013	-0.18; 0.21	0.898
Catania - Palermo - Cagliari	0.068	-0.14; 0.27	0.514
Case vs control	0.053	0.647	0.415
R^2 overall =0.1894 (within = 0.1263; between = 0.2174); Wald χ^2=58.97; p <0.0001			

Model 2 (214 observations, 98 children)	**β**	**95% CI (β)**	**p(Z)**
Outdoor benzene concentration (µg/m^3)	0.009	-0.02; 0.04	0.605
Cotinine (µg/g creatinine)	0.014	0.001; 0.03	0.040
Gender (male vs female)	-0.012	-0.16; 0.14	0.875
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.308	0.08; 0.54	0.008
[4-6) years	0.055	-0.11; 0.22	0.516
Season	Reference Spring		
Summer	-0.040	-0.18; 0.10	0.582
Autumn	0.200	0.04; 0.36	0.012
Winter	0.082	-0.07; 0.24	0.305
Residence	Reference Turin		
Milan	-0.053	-0.28; 0.18	0.657
Florence - Rome	0.048	-0.18; 0.28	0.687
Catania - Palermo - Cagliari	0.003	-0.21; 0.22	0.974
Case vs control	0.011	-0.14; 0.16	0.882
R^2 overall =0.1158 (within = 0.1423; between = 0.0925); Wald χ^2=27.59; p = 0.0063			

Figure 1. Children eligible for inclusion and participation rates



Appendix 1 – Chemical determination: analytical conditions

Benzene concentrations in air samples

The main analytical conditions were the following: desorption at 320 °C for 10 min; overall split ratio 1:75; carrier gas nitrogen at 27 psi; column J&W PONA, 50 m, 0.2 mm id, 0.5 µm film thickness; oven 35 °C for 1 min, 6 °C/min to 110 °C, 20 °C/min to 220 °C, 2 min.

Urine analyses

Pre-treatment and chromatographic conditions used for each analyte are described below.

S-PMA. Pre-treatment of the urine sample (5 mL): calibration curve concentrations = 0, 5, 10, and 50 µg/L; acidification with HCl; centrifugation (10 minutes at 3500 rpm); purification on SPE (Isolute C18 500 mg/3 mL). Chromatographic conditions: Mobile Phase = 60% acetic acid 1% and 40% methanol; Flow = 0.3 mL/min; Column = Symmetry C18 3.0 x 150 mm, 3.5 µm (Waters); Column temperature = 29°C; Run time = 45 min; Volume injected = 21 µL; MS Method = Single Ion Recording of mass 238.0 in ESI neg; LR = 0.3 µg/L.

MA. Pre-treatment of the urine sample (2 mL): calibration curve concentrations: 0, 50, 200, 500, 1000 µg/L; centrifugation (10 minutes at 3500 rpm); purification on SPE (Isolute SAX 500 mg/3mL). Chromatographic conditions: Mobile Phase = 78 % formic acid 0.2 % and 22 % methanol; Flow = 0.3 mL/min; Column= Symmetry C18 3.0 x 150 mm, 3.5 µm (Waters); Column temperature = 30°C; Run time = 30 min; Volume injected = 21 µL. MS Method: Single Ion Recording of mass 141.0 in ESI neg; LR = 7 µg/L.

Cotinine. Pre-treatment of the urine sample (2 mL): calibration curve concentrations: 0, 10, 50, 250, 1000, 3000 µg/L; basification with Ammonium Hydroxide ACS Reagent; centrifugation (10 minutes at 3500 rpm); purification on SPE (Isolute ENV + 50 mg/3mL). Chromatographic conditions: Mobile Phase = 75 % ammonium acetate 3.7mM and 25 % methanol; Flow = 0.3 mL/min; Column = Symmetry C18 3.0 x 150 mm, 3.5 µm (Waters); Column temperature = 30°C; Run time = 33 min; Volume of sample injected = 21 µL. MS Method: Single Ion Recording of mass 177.2 in ESI pos; LR = 0.3 µg/L.

Appendix Table A. Urinary cotinine levels (ln µg/g of creatinine) by smoking habits of the parents, gender, age, season, province of residence, and caseness (295 observations from 95 children)

	β	95% CI (β)	p(Z)
Parental smoking habits	Reference Nonsmokers		
One parent smoking	0.852	0.50; 1.20	<0.001
Both parents smoking	1.685	1.22; 2.15	<0.001
Gender (male vs female)	0.028	-0.31; 0.37	0.872
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.214	-0.22; 0.65	0.338
[4-6) years	0.111	-0.27; 0.49	0.566
Season	Reference Spring		
Summer	-0.193	-0.43; 0.05	0.116
Autumn	-0.015	-0.26; 0.23	0.901
Winter	0.260	0.02; 0.50	0.035
Residence	Reference Turin		
Milan	-0.348	-0.90; 0.20	0.215
Florence - Rome	0.636	0.14; 1.13	0.011
Catania - Palermo - Cagliari	0.511	0.002; 1.02	0.049
Case vs control	0.229	-0.09; 0.55	0.164
R^2 overall =0.4213 (within = 0.0732; between = 0.5150); Wald χ^2=110.31; p<0.0001			

Appendix Table B. Participation bias factors calculated using different cut-points to dicothomize outdoor benzene concentrations

Cut-point = P50 = 3.25 μg/m^3		Exposed	Not Exposed	Bias factor
Cases	Participant	18	25	1.03
	Non Participant	11	12	
Controls	Participant	28	28	
	Non Participant	44	36	
Cut-point = P75 = 4.34 μg/m^3		Exposed	Not Exposed	Bias factor
Cases	Participant	4	39	0.64
	Non Participant	7	16	
Controls	Participant	14	42	
	Non Participant	26	54	
Cut-point = 5 μg/m^3		Exposed	Not Exposed	Bias factor
Cases	Participant	3	40	1.42
	Non Participant	4	19	
Controls	Participant	4	52	
	Non Participant	16	64	

Appendix Table C. Relationship between estimated exposures to ELF-MF (48 h TWA in the child's bedroom, ln μT) and to outdoor benzene (individual averages of repeated seasonal measurements, μg/m^3), controlling for gender, age, province of residence, caseness, and participation in the benzene pilot study (125 observations; 48 cases and 77 controls)

	β	95% CI (β)	p (t)
Outdoor benzene (μg/m^3)	0.177	0.06; 0.29	0.002
Gender (male vs female)	-0.332	-0.74; 0.08	0.112
Age (at diagnosis)	Reference [6-10] years		
[0-2) years	0.120	-0.56; 0.80	0.728
[2-4) years	0.166	-0.38; 0.72	0.550
[4-6) years	0.334	-0.29; 0.96	0.295
Residence	Reference Turin		
Milan	-0.007	-0.65; 0.64	0.984
Florence-Rome	0.135	-0.50; 0.76	0.673
Catania-Palermo-Cagliari	0.521	-0.13; 1.17	0.116
Case vs control	-0.024	-0.43; 0.38	0.908
Participant vs non participant	0.520	0.09; 0.95	0.019
F (10, 114 df) = 2.13; prob > F = 0.0271; R^2 = 0.1577			

STROBE Statement—Checklist of items that should be included in reports of ***case-control studies***

	Item No	Recommendation
Title and abstract	1★	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2★	Explain the scientific background and rationale for the investigation being reported
Objectives	3★	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4★	Present key elements of study design early in the paper
Setting	5★	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6★	(*a*) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls
		(*b*) For matched studies, give matching criteria and the number of controls per case
Variables	7★	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*★	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9★	Describe any efforts to address potential sources of bias
Study size	10★	Explain how the study size was arrived at
Quantitative variables	11★	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12★	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, explain how matching of cases and controls was addressed
		(*e*) Describe any sensitivity analyses
Results		
Participants	13*★	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*★	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
Outcome data	15*★	Report numbers in each exposure category, or summary measures of exposure
Main results	16★	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period

Other analyses	17★	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18★	Summarise key results with reference to study objectives
Limitations	19★	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20★	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21★	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22★	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

Exposure to benzene and childhood leukaemia: a pilot case-control study

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Exposure to benzene and childhood leukaemia: a pilot case-control study

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Keywords: acute lymphoblastic leukaemia, benzene, extremely low frequency magnetic fields (ELF-MF), biomarkers, children, participation bias, confounding, epidemiologic methods.

Word count: 4654

ABSTRACT

Objectives

Main purpose: to evaluate the feasibility of a measurement-based assessment of benzene exposure in case-control studies of paediatric cancer.

Additional aims: to identify the sources of exposure variability; to assess the performance of two benzene biomarkers; to verify the occurrence of participation bias; to check whether exposures to benzene and to 50 Hz magnetic fields were correlated, and might exert reciprocal confounding effects.

Design

Pilot case-control study of childhood leukaemia and exposure to benzene assessed by repeated seasonal weekly measurements in breathing zone air samples and outside the children's dwellings, with concurrent determinations of cotinine, *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) in urine.

Participants

108 cases and 194 controls were eligible for inclusion.

Results

Full-participation was obtained from 46 cases and 60 controls, with low dropout rates before 4 repeats (11% and 17%); additional 23 cases and 80 controls allowed collection of outdoor air samples only.

The average benzene concentration in personal and outdoor air samples was 3 $\mu g/m^3$ (SD 1.45) and 2.7 $\mu g/m^3$ (SD 1.41), respectively.

Personal exposure was strongly influenced by outdoor benzene concentrations, higher in the cold seasons than in warm seasons, and not affected by gender, age, area of residence, or caseness.

Urinary excretion of S-PMA and personal benzene exposure were well correlated.

Outdoor benzene levels were lower among participant controls compared to non-participants, but did not differ between participant and non-participant cases; the direction of the bias was found to depend on the cut-point chosen to distinguish exposed and unexposed.

Exposures to benzene and ELF-MF were positively correlated.

Conclusions

Repeated individual measurements are needed to account for the seasonal variability in benzene exposure, and have the additional advantage of increasing the study power. Measurement-based assessment of benzene exposure in studies of childhood leukemia appear feasible, although financially and logistically demanding.

ARTICLE SUMMARY

Article focus

- Benzene is an established cause of acute non lymphocytic leukaemia (AnLL), and there is limited evidence for an association between exposure to this agent and other hematologic neoplasms. Epidemiologic studies of benzene and childhood leukemia have provided inconsistent results, possibly due to the use of surrogate exposure proxies, and lack of analyses by leukaemia subtype.
- Our pilot study was aimed at evaluating the logistic feasibility of an assessment of benzene exposure based on repeated measurements in a case-control study of childhood leukemia. A few methodological issues were also addressed (putative determinants of exposure variability; performance of urinary levels of MA and S-PMA as benzene biomarkers in children; participation bias; possible reciprocal confounding effects of exposures to benzene and to ELF-MF).

Key messages

- Eligible for inclusion were 108 cases and 194 matched controls, aged 2 to 12 years at the time of the survey. Full participation rates were low, , but the outdoor monitoring was accepted by 64% of cases and 72% of controls . Adherence of full participants to the scheduled repeats was very satisfactory (cases 89%, controls 83%).
- Personal exposure was strongly influenced by outdoor benzene concentrations, was higher in the cold seasons than in warm seasons, and was not affected by gender, age, area of residence, or caseness. Personal benzene exposure and urinary excretion of S-PMA (but not of MA) were well correlated. A participation bias was indeed present. A positive association between exposures to benzene and ELF-MF was observed.
- Epidemiologic studies of paediatric cancer and estimates of environmental benzene exposure based on repeated seasonal measurements, although challenging, appear logistically feasible.

Strengths and limitations

- To our knowledge, this is the first pilot study of childhood leukaemia and measured personal benzene exposure.

- The study size is very small. The greater accuracy of measurement-based exposures estimates, compared to surrogate exposure proxies, does not necessarily correspond to increased validity, especially when measurements are used for retrospective post-diagnosis exposure assessments.

INTRODUCTION

Benzene is a ubiquitous air pollutant, that needs to be metabolized to become carcinogenic.[1- 2]

Benzene exposure and acute non lymphocytic leukaemia (AnLL) are causally related in adult humans, while there is limited evidence for an association between exposure to this agent and acute or chronic lymphocytic leukaemia, multiple myeloma, and non-Hodgkin's lymphoma.[3] Moreover, a dose-dependent association between benzene exposure and incidence of myelodysplastic syndrome has been observed among petroleum workers. [4]

Exposure to benzene would increase the risk of AnLL at levels of ≥40 ppm-years of occupational cumulative exposure, equivalent to a lifetime (76 years) environmental exposure of ≥120 ppb.[5]

Due to the established carcinogenicity of benzene, WHO has not developed any guideline value for this chemical in air, while indicating that ambient benzene concentrations of 17, 1.7 and 0.17 $\mu g/m^3$ are associated with excess lifetime risks of leukaemia of 10^{-4}, 10^{-5} and 10^{-6}, respectively.[6-7]

While it seems unlikely that benzene is a major cause of leukaemia in the general population exposed in the ppb range, children may represent a subpopulation with increased susceptibility.[1, 3]

Childhood leukaemias have distinctive features compared to leukaemias in adults. The major subtypes are acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML), accounting for 80% and 15% of cases aged 0 to 14 years in white populations respectively.[8] Both subtypes are thought to develop through a first initiating event *in utero* (e.g. the TEL-AML1 gene fusion whose prevalence in newborns has been estimated at around 1% while it is observed in 25% of ALL cases) followed by further postnatal genetic changes.[8] The "second hit" might consist of additional idiopathic chromosomal translocations, as well as of exposures to biological, chemical

or physical agents.[9] Ionizing radiation, benzene, alkylators and topoisomerase II inhibitors are among the few confirmed environmental risk factors for AML, while delayed, dysregulated responses to common infections are likely to play a major role in the conversion of pre-leukemic clones into overt ALL.[8-9]

Findings from available studies of benzene and childhood leukaemia are inconsistent, possibly due to the use of indirect estimates of exposure and lack of analyses by leukaemia subtype.[10]

To advance current understanding of benzene health effects and susceptibility, studies of paediatric cancers that include estimates of environmental exposure to benzene, rather than surrogate exposure indicators, have been recommended.[11]

Major challenges in pursuing this suggestion include the space- and time-variability of ambient benzene levels, the low exposure levels in children, and the inherent susceptibility of case-control studies (the design of choice for etiological studies of rare disease like childhood cancer) to selection and information bias.

We evaluated the logistic feasibility of an assessment of benzene exposure based on repeated seasonal weekly measurements in breathing zone air samples and outside the children's dwellings, with concurrent determinations of cotinine, *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) in urine, in a pilot investigation within an Italian case-control study on environmental risk factors for childhood leukaemia (SETIL).

Additional objectives of the pilot study were:

- to investigate the relationship between level personal exposure to benzene and putative determinants (atmospheric benzene, second-hand tobacco smoke, individual traits);
- to assess the performance of *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) as benzene biomarkers in children;

- to verify the occurrence of participation bias from differential adhesion to the benzene measurement study, and estimate the amount and direction of the distortion;
- to check whether exposures to benzene and to extremely low frequency magnetic fields (ELF-MF) were correlated, and might eventually exert reciprocal confounding effects on the relationship with childhood leukaemia.

METHODS

Study population

Incident cases of childhood leukaemia from 14 Italian regions, aged 0 to 10 years at diagnosis in 1998-2001, were eligible for enrolment in the SETIL study. Cases were ascertained through the national registry run by the Association of Paediatric Haematology and Oncology (AIEOP). Controls, matched to cases (2:1 ratio) on gender, date of birth, and region, were randomly selected from population lists. Information on several items concerning the children, their next-of-kin and dwellings, was collected by interview of parents. All interviewed families were invited to participate in a measurement study of indoor ELF-MF, while subsets of participants were asked to join two side-investigations, on exposure to gamma radiation and benzene, respectively.

Eligibility for the benzene pilot study was restricted to 108 childhood leukaemia cases from seven Italian provinces (Turin, Milan, Florence, Rome, Catania, Palermo, and Cagliari), diagnosed between July 2000 and December 2001, and 194 matched controls.

The study protocol was approved by the Piedmont Ethical Committee on 14 January 2002.

Sampling strategy and devices

Due to the high daily and seasonal variability of atmospheric benzene concentrations, the protocol called for four repeated seasonal one-week samplings of breathing zone air per child over one

year ("personal" air samples), with concurrent collection of urine samples and atmospheric air samples in proximity of the children's homes ("outdoor" air samples).

Outdoor air sampling would also be performed, with an identical strategy, near the homes of all eligible non-participants.

To study the day-to-day variability in exposure, 24-h repeated personal and indoor samples during four season-specific weeks would be collected from a subset of children and related homes.

Personal air samples were collected by passive samplers (Radiello® radial symmetry diffusive sampler) worn by the child during the day and placed at the bedside at night.

Radiello® samplers were also used to collect outdoor air samples, placed near the entrance of the dwellings (within 1 meter), at a vertical distance from the ground suitable to avoid infringements (2-2.5 m), stored in a plastic case to avoid rain or snow.

At retrieval, the adsorbing cartridges were removed from the diffusive bodies and placed into glass storage tubes. The ID code of the child, along with dates and times of sampling start and end, were recorded on self-adhesive labels stuck on the tubes. The cartridges were sent to a single laboratory (Fondazione Salvatore Maugeri, Padova) for the chemical analyses.

Daily urine samples (10 ml, from the last micturition before sleep) were collected for 7 subsequent days (70 ml per week) during each seasonal survey. The daily samples were pooled in one plastic vial, and kept in the freezer compartment of the home refrigerator until collection at the end of the week. The vials were transported to the local research centre in cool bags, and stored at –5 °C until delivery (packed in dry ice and usually in 2 weeks) to the laboratory (Fondazione Salvatore Maugeri, Pavia).

Field work began between March 2002 and January 2003, and ended in October 2003 - July 2004, depending on the local research centre.

Chemical determinations

Benzene concentrations in air sample were determined by an automated thermal desorber (ATD400, Perkin Elmer) coupled to a capillary gas-chromatography system (Autosystem XL, Perkin Elmer). The expanded uncertainty of the method, in the range 2.4 to 14.3 μg/m^3, was shown to be 18%.[12] The limits of detection and quantification, over 1 week exposure, are 0.05 μg/m^3 and 0.1 μg/m^3.

The urine analyses were performed using a high pressure liquid chromatography system (Alliance 2690, Waters) equipped with a spectrometric (SM) detector (ZQ, Waters) following a preliminary step of purification of the samples on pre-activated solid phase extraction (SPE) cartridges. The limit of detection (LOD), coefficient of variation (CV) and accuracy of the method were: LOD = 1 μg/L, CV % = (1.22)-(1.10), accuracy % = (- 2.39)-(3.36) for S-PMA; LOD = 20 μg/L, CV % = (1.33)-(1.06), accuracy % = (- 2.18)-(3.27) for MA; LOD = 1 μg/L, CV % = (1.25)-(1.09), accuracy % = (-2.29)-(3.33) for cotinine.

Further details are provided in Appendix 1.

The chemical determinations were completed by May 2005.

Statistical analyses

Measurements below the chemical-specific detection limits were assigned half such values and included in the analyses.

The relationships between personal exposure to benzene and putative determinants (as well as between urinary excretion of benzene metabolites, benzene intake, and other covariates) were assessed by generalized least squares (GLS) models for repeated measurements (STATA v. 11, xtreg procedure). The GLS model is: $y_{it} = \alpha + X_{it}B + u_{it} + e_{it}$, where i (1 to n) is the number of observations collected at time t (1 to 4) and u_{it} and e_{it} are the error components.

As concentrations of benzene and urinary analytes were log-normally distributed, we always included in the models log-transformed dependent variables.

We used the odds ratio (OR), calculated from generalized estimating equations (GEE) for repeated individual measurements (STATA v. 11, procedure xtgee), to estimate the association between benzene exposure and dichotomous variables such as case-control or participation status. The general equation of the GEE model is $g\{E(y_j)\}=x_j\beta$, where g is the link function, herein a logit function.

We calculated a participation bias factor following the method suggested by Greenland [bias factor = $(S_{1a}*S_{0b})/(S_{0a}*S_{1b})$], where S_{1a}, S_{0a}, S_{1b}, and S_{0b} denote the probabilities of selection (i.e. full participation in the benzene study) for exposed cases, unexposed cases, exposed controls, and unexposed controls.[13] When the bias factor equals 1, there is no bias, when it is above or below 1 the true OR will be biased respectively upward or downward by the magnitude of this factor.

Multiple regression models were used to analyze the relation between estimated exposures to benzene and ELF-MF.

RESULTS

Participation and sampling outcome

Out of 108 cases and 194 controls eligible for inclusion, 46 cases and 60 controls (43% and 31%) agreed to take full part in the benzene side-study (Figure 1).

In addition, the parents of 23 cases and 80 controls who refused the personal exposure assessment accepted the outdoor monitoring (partial participation = 21% and 41%).

Altogether 1467 air samples were collected. A small percentage (2%) were lost during monitoring (22 samplers stolen, 2 sampler plates broken, 3 cartridges lost), transport (8 missing labels) or

chemical analysis (2 cartridges broken on arrival at the laboratory; 1 sample lost due to equipment failure).

Benzene measurements from the day-to-day variability sub-study (19% of the total) could not be used because only four control children accepted the 24-h sampling scheme, and were replaced by the calculated weekly averages.

A further 20% of benzene measurements was removed from the data-set due to lack of compliance with the study protocol (indoor samples collected in place of the personal ones from children refusing to wear the sampler; time-or place-mismatch of personal and outdoor samples; "orphan" personal or outdoor samples; duplicate season-specific measurements; non-participants replaced with children ineligible for the benzene side-study].

For the same reasons, 107 out of 417 chemical determinations in urine (26%) were discarded.

Three cases and 5 controls were excluded from one or more analyses due to lack of complete measurement sets in all seasonal series and, although 89% and 83% of full-participant cases and controls did adhere to all four seasonal surveys, only 37% and 43% of them had four repeated analyzable observations.

Personal characteristics of the children

The families of cases participating in full to the benzene study had been interviewed on average 1.3 years (SD 0.47) after the date of diagnosis, and the control-families 1.5 years (SD 0.46) after the corresponding reference date. The delay between diagnosis and the first series of benzene measurements was 2 years (SD 0.53) for both cases and controls.

Cases and controls were comparable in terms of gender, age, and father's attained educational level (Table 1). A higher proportion of controls than cases had both parents smoking, and control-mothers were more educated than case-mothers. There were similar proportions of only children

in the case and control groups, while firstborn children were more frequent among controls than cases. Early schooling (day-care attendance) was more common in cases than in controls. At the time of the benzene survey, most children were still living in the home occupied at birth or in the house they moved into after birth but before the date of diagnosis (cases 95%; controls 91%).

Level, variability, and determinants of personal exposure to benzene

The analyses of level, variability and determinants of personal exposure to benzene were based on 43 cases (39 ALL and 4 AML) and 56 controls, with 261 valid pairs of benzene concentrations in breathing zone and outdoor air (110 from cases and 151 from controls). A large proportion of these children (35%) had a single pair of concurrent measurements, unevenly distributed by season, with a disproportionally high number of summer samples (30 out of 35, all but one from a single centre).

The distributions, overall and by season, of benzene concentrations in personal and outdoor air samples, and of cotinine, MA and S-PMA in urine are described in Table 2.

Personal exposure to benzene was log-normally distributed (Shapiro-Wilk test = 0.938, $p<0.001$), and the mean benzene level over the individual yearly averages was 3 $\mu g/m^3$ (0.92 ppb).

The distribution of benzene outdoor concentration was skewed to the left in all seasons and the yearly averages were log-normally distributed as well (Shapiro-Wilk test = 0.948, $p = 0.001$); the average yearly benzene level near the children's homes was 2.7 $\mu g/m^3$ (0.83 ppb).

Both outdoor benzene concentrations and personal exposure levels were higher in the cold seasons (autumn-winter) than in the warm ones (spring-summer).

The European limit for benzene in air (5 $\mu g/m^3$) was exceeded by 5% of the yearly average outdoor concentrations, and by 8% of the yearly average levels in breathing zone air samples. A large

proportion of autumn and winter measurements were above 5 μg/m^3 (35% and 25% outdoor; 26% and 30% of the personal exposure estimates).

Cases and controls had similar levels of personal exposure to benzene: the leukaemia OR for a unit increase (1 μg/m^3) in personal benzene exposure was 0.93 (95% CI 0.77-1.13) adjusting for gender, age at the benzene survey (2-4; 4-6; 6-12 years), cotinine in urine (μg/g creatinine), season, and province of residence (Turin; Milan; Florence - Rome; Catania - Palermo - Cagliari).

A similar lack of association was found between the odd of disease and benzene concentration outside the children's homes [OR 0.94 (95% CI 0.80-1.09)], controlling for gender, age, smoking habits of the parents at the interview (non-smokers, mother or father smoking; both parents smoking), season, and province of residence.

Further adjustment for birth order and age at first schooling had no material effect on the observed leukaemia-benzene relationship [personal exposure: OR 0.92 (95% CI 0.75-1.13); outdoor benzene: OR 0.95 (95% CI 0.81-1.13)].

As cases and controls had comparable levels of benzene exposure, we carried out the analyses illustrated in the forthcoming paragraphs on the whole data-set, although always controlling for caseness.

Urinary cotinine concentration (μg/g of creatinine) was higher in children of smoking parents compared to children of non-smokers, and children with both parents smoking excreted a larger amount of cotinine than children with one parent smoking (Appendix Table A). Cotinine levels were higher in winter than in other seasons, and higher in children from central and southern Italy (Florence, Rome, Palermo, Catania, Cagliari) than in children from northern provinces (Turin and Milan). The high between- *vs* within-subject R^2 ratio is worth noting.

Personal benzene exposure was strongly influenced by outdoor benzene concentrations (Table 3-A), and apparently not affected by gender or age; the season showed a modifying effect, with increasing levels of personal exposure during autumn and winter; the fraction of variability explained by the model was higher for the within-subject component than for the between-subject one.

Exposure to second-hand tobacco smoke (estimated by cotinine excretion or by parental smoking habits) showed a trivial influence on personal exposure to benzene. The inclusion of urinary cotinine (μg/g creatinine) in the model described in Table 3-A, slightly decreased its goodness of fit [R^2 overall = 0.46; Wald χ^2=189.49; R^2 within = 0.55; R^2 between = 0.35; β (cotinine) = 0.012; 95% CI = -0.003; 0.03)]; an alternative model, including smoking habits of the parents, did not perform any better [R^2 overall = 0.46; Wald χ^2=216.44; R^2 within = 0.52; R^2 between = 0.39; β (one parent smoking) = 0.14; 95% CI = -0.02; 0.31; β (both parents smoking) = 0.17; 95% CI = -0.06; 0.39].

Children from central Italy (Florence and Rome) tended to have lower benzene concentrations in breathing zone air samples compared to residents in other provinces, all other things being equal (Table 3-A), possibly because of residual confounding from lack of samples collected in Rome other than in summer. We tried to verify this hypothesis by restricting the analyses to children with at least two series of measurements in different seasonal periods (cold and warm). The data-set reduced to 61 subjects (25 cases and 36 controls) and 220 pairs of personal-outdoor benzene measurements. Actually, children from Florence still showed (not significantly) lower levels of personal exposure to benzene (β = - 0.27; 95% CI = -0.56; 0.03; p =0.074) compared to children from Turin. In the restricted data-set, however, independent effects of both outdoor benzene and urinary cotinine levels on personal benzene exposure were observed (Table 3-B).

Benzene intake and urinary excretion of benzene metabolites

Ninety-eight children (43 cases and 55 controls) and 310 pairs of urine and breathing zone air measurements (138 from cases and 172 from controls) were available for the analyses of the urinary excretion of benzene metabolites (MA and S-PMA) in relation to personal exposure to benzene.

Urinary concentrations of S-PMA (ln µg/g creatinine) were related to personal exposure to benzene (Table 4, Model 1). Youngest children (2-4 years at the benzene survey) excreted higher level of S-PMA compared to children aged 6-12 years, all other conditions being equal, and urinary concentration of S-PMA were higher in samples collected during the cold seasons compared to spring samples. The model, however, explained just 19% of the overall S-PMA variability. In an alternative model, including outdoor benzene concentrations and urinary cotinine in place of personal benzene exposure, we also observed an effect of the nicotine biomarker on S-PMA excretion (Table 4, Model 2).

On the contrary, neither benzene concentrations in breathing zone air samples, nor outdoor benzene concentrations or cotinine levels explained the intra- and inter-individual variability in urinary levels of MA, controlling for gender, age, season, area of residence, and caseness (data not shown).

Bias due to differential participation

Available for the analysis of participation bias were 66 cases (43 full-participant and 23 partial-participant) and 136 controls (56 and 80), with 652 measurements of outdoor benzene concentrations (135 and 175 from full-participant cases and controls; 81 and 261 from partial-participant cases and controls).

Benzene concentrations near the homes of full-participant controls were significantly lower than those in proximity of partial-participants' dwellings (OR = 0.88; 95% CI 0.80-0.97), adjusting for

gender, age, season and place of residence, while there was no difference in ambient benzene levels between participant and non-participant cases (OR = 0.95; 95% CI 0.82-1.09). As participation in the study was also associated with the case-control status, assuming a causal association between exposure and disease, a selection bias might ensue. However, as parents of more exposed controls were less willing to accept to be interviewed, an upward distortion would be expected, which is at odds with the apparent lack of association between personal benzene exposure and leukaemia risk in the current study.

To the aim of the current analysis, personal exposure to benzene was dichotomized around the median (3.25 μg/m^3), the 75th percentile (4.34 μg/m^3) or 5 μg/m^3 (the current limit for airborne benzene in Europe). The amount and direction of bias were found to depend on the cut-point chosen (Appendix Table B), whereas no bias is expected when the exposure is categorized around the median (bias factor = 1.03), and biases in the opposite directions are predicted using cut-off at p75 and at 5 μg/m^3 (0.64 and 1.42, respectively).

Relationship between exposures to benzene and ELF-MF

Children with benzene and ELF-MF measurements made at the same house qualified for inclusion in the analysis of the relationship between estimated exposures to these agents. As only 35 cases and 46 controls met such criterion when benzene concentrations in breathing zone air samples were used as exposure indicator, we performed the analysis on 48 cases and 77 controls with place-comparable pairs of average yearly outdoor benzene concentration (μg/m^3) and 48 h TWAs of ELF-MF level in the child's bedroom (ln μT).

There was a positive association between estimated exposures to ELF-MF (dependent variable) and benzene (β = 0.177; 95% CI 0.06-0.29; p = 0.002); the multivariable regression model (including gender, age, province of residence, caseness, and participation in the benzene pilot study as covariates) explained 16% of the variability in the dependent variable [F (10, 114 df) =

2.13; p> F = 0.0271]. A steeper increase in ELF-MF level per unit increase in outdoor benzene concentration (β = 0.520; 95% CI 0.09-0.95; p = 0.019) was seen among the 81 children fully participating in the benzene pilot-study compared to the 44 partial-participants (Appendix Table C).

Similar results, with a more accentuated increase in indoor magnetic induction level per unit increase in outdoor benzene concentration [β= 0.272; 95% CI = 0.09-0.45; p(t) = 0.003; R^2 = 0.19], were observed in the restricted data-set of 86 children with ≥2 weekly samplings in alternate seasons.

DISCUSSION

We have carried out a pilot case-control study of childhood leukaemia and exposure to benzene assessed by repeated individual measurements made on average two years after diagnosis. The pilot study included side-investigations aimed at evaluating the performance of two biological indicators of benzene exposure in children, at estimating amount and direction of a possible participation bias, and at assessing the relation between estimated exposures to benzene and ELF magnetic fields.

Due to the relatively low incidence of childhood cancers (10-15 for 100,000 person-years in the 0-14 year range in most industrialized countries), the case-control approach is the design of choice for analytical epidemiologic studies about potential risk factors for these diseases. Such a study design, however, is inherently prone to measurement errors stemming from the retrospective reconstruction of the exposures of interest, and to differential participation leading to control samples not representative of the study base. Therefore, findings from observational epidemiologic studies of postulated determinants for childhood malignancies are often inconsistent and always require a cautious and thoughtful interpretation.[14]

Although based on small numbers, some of the findings from the current study have a certain factual and methodological interest.

Repeated samplings of breathing and outdoor air are indeed needed to account for the seasonal variability in environmental benzene levels.[15-16]

On average, children participating in the current study appear to experience mean yearly levels of personal exposure to benzene not exceeding the European guidelines (although 8% percent of the yearly mean levels were above 5 μg/m^3).

What we *a priori* considered the main sources of benzene exposure for children (ambient benzene levels and second-hand tobacco smoke) explained no more than half of the overall variability in personal exposure, which indicates the need to identify other sources of exposure particularly relevant, perhaps, during the cold seasons. In fact, in autumn-winter compared to spring-summer, we observed higher levels of personal exposure to benzene, of urinary cotinine and of S-PMA excretion, all other things being equal. These findings might be due to the lower ventilation rates in homes and schools during the cold seasons, to winter-specific sources of indoor benzene concentrations not considered in the current survey (e.g. fireplaces or other combustion sources), and/or to the seasonal variability in daily patterns of time spent in different micro-environments (e.g. within cars or buses).[17]

Some case-control studies have suggested an association between exposure to traffic density and childhood leukaemia;[18-21] however, negative findings have also been reported.[22-25] Positive associations between incidence of ALL in children and residential proximity to petrol stations were observed in three case–control studies.[23, 26-27] An increased risk of childhood leukaemia in relation to estimated exposure to benzene was observed in a small Italian study,[28] but not in a much larger case-control study carried out in Denmark and based on a sophisticated and validated exposure modelling.[29]

To our knowledge there is no previous study of childhood leukaemia and measured personal benzene exposure. Moreover, as only children aged 0 to 10 years at diagnosis were eligible for the SETIL study, the large majority of cases included in the current investigation were pre-B ALL.

Cases and controls did not differ in terms of exposure to benzene, estimated either by benzene level in personal air samples or through outdoor benzene concentration, but the interpretation of this finding is hampered by the retrospective exposure assessment and the low statistical power of this preliminary investigation. That notwithstanding, due to the design based on repeated individual observations, the risk estimates have quite narrow confidence intervals. Thus the findings from this pilot study, in accordance with the limited evidence for an association between exposure to benzene and ALL,[3, 5] might also suggest that the levels of benzene exposure experienced by children living in Italian towns do not entail a detectable increase in the risk of ALL.

Current perspectives on the causes of childhood ALL increasingly point towards an etiologic role of altered patterns of infections and related immune stimulation during the first years of life, and one piece of supporting evidence is the consistent observation of an inverse association between ALL risk and day-care attendance.[30] Studies of childhood ALL and birth order, on the other hand, have provided inconsistent result.[31] Neither age at first schooling, nor birth order confounded the relation between childhood leukaemia and indicators of benzene exposure in the current study.

S-PMA concentration measured in repeated weekly samples of the last micturition before sleep was found to reflect personal exposure to benzene, although the available covariates explained a small fraction of the within- and between-subject variability of this benzene metabolite. This is a quite surprising result, considering that S-PMA is believed to represent less than 1% of urinary benzene metabolites for exposures to benzene at air concentrations between 0.1 and 10 ppm.[32]

Benzene exposure proved not able to explain the variability of MA urinary excretion observed in our children, consistent with findings from a previous Italian study.[33] The low statistical power of the study, the low level of benzene exposure, and the lack of adjustment for the confounding effect of dietary intake of sorbic acid (a common food additive), may explain this finding.[34]

Full-participation rates were low, in line with a general tendency to decreasing participation rates, especially in epidemiological studies requiring adherence to complex measurement protocols.[14, 35] That notwithstanding, the outdoor monitoring was accepted by a fairly satisfactory proportions of families (64% and 72% of eligible cases and controls). This is an encouraging result, given the strong correlation between personal benzene exposure and ambient benzene level observed in the current study.

We observed a differential participation bias, which underscores the need to plan parallel bias analyses in any case-control study.[36] The dependence of the participation bias factor on the cut-point chosen to dichotomize the exposure variable is of methodological interest.

The positive association between the 48 h TWA of ELF-MF induction in the child's bedroom and the average yearly concentrations of outdoor benzene will need consideration in the interpretation of findings from the analyses of childhood leukaemia risk in relation to 50 Hz MF in the SETIL case-control study.

Incidental failures during sample collection, transport or chemical analysis accounted for a negligible proportion of lost air or urine samples. However, substantial percentages of chemical measurements could not be included in current analyses because of misunderstanding of the sampling protocol.

The day-to-day variability sub-study was clearly too demanding to be acceptable.

In conclusion, the current pilot study suggests that epidemiologic studies of childhood leukaemia risk and measurement-based estimates of exposure to benzene are challenging but logistically feasible (provided that the study protocol specifies every single sampling detail and nothing is considered so obvious as to be omitted). Such an exposure assessment method could be considered by epidemiologists willing to involve in the "genome - exposome" approach to gain further insight into the relationship between benzene exposure and childhood leukaemia risk, with priority given to AML.[2, 37-39] Due to the low incidence rates of AML in children, however, international multi-centre studies are needed to address this topic.

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COMPETING INTERESTS

None.

CONTRIBUTORSHIP

Susanna Lagorio designed the study, planned the statistical analyses, and drafted the manuscript.
Daniela Ferrante carried out the statistical analyses.
Alessandra Ranucci was in charge of the data management, quality control and descriptive statical analyses.
Paolo Sacco and Sara Negri collaborated to the study design, and were responsible for the

chemical analyses.
Roberto Rondelli, as manager of the AIEOP childhood leukaemia registry, performed the case ascertainment.
Santina Cannizzaro, Valeria Torregrossa, Pierluigi Cocco, Francesco Forastiere, Lucia Miligi, Luigi Bisanti, and Corrado Magnani were the principal investigators of the local centres collaborating to the benzene pilot study in the framework of the SETIL multicentre case-control study.
All the authors critically revised the early drafts, collaborated to the discussion of the study findings, and approved the final version of the manuscript.

DATA SHARING

Additional explanatory material is available to everyone on request. The dataset is available to fellow researchers for further joint analyses, on request to the corresponding author, and pending approval by the co-authors.

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Table 1. Children included in the pilot study by selected characteristics

		Cases		Controls	
		N	%	N	%
Gender	Female	25	58	30	54
	Male	18	42	26	46
Age at the survey	[2,4) years	5	12	9	16
	[4,6) years	21	49	16	29
	[6,12] years	17	40	31	55
Residence*	Turin	7	16	9	16
	Milan	8	19	13	23
	Florence	3	7	5	9
	Rome	14	33	15	27
	Catania	3	7	5	9
	Palermo	4	9	6	11
	Cagliari	4	9	3	5
Parent smoking§	None	20	47	27	48
	One	16	37	18	32
	Both	4	9	11	20
	Missing	3	7	0	-
Father's education§	No qualification	-	-	1	2
	Primary school	17	40	21	38
	High school	17	40	24	43
	University degree	6	14	10	18
	Missing	3	7	-	-
Mother's education§	No qualification	-	-	-	-
	Primary school	19	44	17	30
	High school	15	35	26	46
	University degree	9	21	13	23
	Missing	-	-	-	-
Birth order§	Only child	10	23	12	21
	First born	10	23	20	36
	Second born or higher birth order	23	53	24	43
Age at first schooling§	No schooling yet	15	35	16	29
	<3 years (crèche)	14	33	9	16
	[3,6) years (preschool)	14	33	30	54
	[6-7] years (primary school)	0	-	1	2
Home at the time of the benzene survey^	Occupied since birth	28	65	39	70
	Moved into after birth & before diagnosis	13	30	12	21
	Moved into after diagnosis & before interview	1	2	5	9
	Moved into after interview	1	2	-	-
Total		43	100	56	100

*At the time of diagnosis or the corresponding reference date for controls; §Information reported at the interview; ^The ELF magnetic fields measurements, if the parents agreed, were made at the time of the interview.

Table 2. Benzene concentration in personal and outdoor air samples, and urine level of cotinine and benzene metabolites by season and overall

	Obs (#)	Mean	SD	G-mean	G-SD	Min	Percentiles			Max
Benzene in personal air samples (µg/m^3)							**p25**	**p50**	**p75**	
Spring	57	2.51	1.89	2.10	1.75	0.60	1.50	1.82	3.11	11.12
Summer	86	2.26	1.45	1.90	1.82	0.47	1.25	1.85	3.10	8.13
Autumn	62	4.31	2.60	3.73	1.57	0.92	2.939	3.70	5.17	18.47
Winter	56	4.04	1.78	3.67	1.73	1.55	2.34	4.00	5.24	9.03
Individual yearly averages	99	3.00	1.45	2.66	1.67	0.75	2.05	2.90	3.83	9.00
Benzene in outdoor air samples (µg/m^3)										
Spring	57	2.29	1.30	1.93	1.84	0.48	1.20	1.91	3.15	5.67
Summer	86	1.94	1.20	1.65	1.75	0.39	1.12	1.58	2.28	6.92
Autumn	62	3.99	2.58	3.05	1.92	0.08	1.93	3.42	5.63	11.18
Winter	56	3.80	1.86	3.25	2.35	0.15	2.40	3.66	5.20	8.31
Individual yearly averages	99	2.70	1.41	2.33	1.78	0.27	1.59	2.37	3.63	6.92
Cotinine (µg/ g creatinine)										
Spring	78	3.92	7.04	1.91	3.26	0.05	1.00	1.94	3.50	49.0
Summer	78	3.20	5.52	1.50	3.59	0.09	0.82	1.68	3.71	41.4
Autumn	76	4.54	8.51	1.92	3.92	0.05	1.20	1.93	4.30	48.7
Winter	74	4.36	7.38	2.32	3.01	0.10	1.20	2.30	4.80	53.5
Individual yearly averages	98	3.73	5.99	2.14	2.67	0.30	1.08	2.09	3.58	41.9
MA (µg/g creatinine)										
Spring	81	104.22	69.28	87.43	1.79	17.00	60.27	82.00	126.99	349.00
Summer	79	140.40	226.73	92.30	2.16	13.33	56.54	83.00	131.76	1680.00
Autumn	76	128.24	124.04	99.57	1.94	30.21	60.16	102.48	147.21	893.04
Winter	74	119.09	100.15	95.30	1.86	26.00	65.00	86.00	129.00	591.00
Individual yearly averages	98	116.65	84.89	101.06	1.62	46.42	73.33	92.66	122.50	593.42
S-PMA (µg/g creatinine)										
Spring	81	1.13	0.60	1.00	1.62	0.21	0.80	1.00	1.30	3.70
Summer	79	1.12	0.54	1.02	1.54	0.41	0.72	1.00	1.39	3.30
Autumn	76	1.53	0.93	1.33	1.67	0.49	0.97	1.29	1.84	5.80
Winter	74	1.37	0.60	1.23	1.64	0.15	1.00	1.20	1.60	3.40
Individual yearly averages	98	1.28	0.50	1.20	1.43	0.56	0.94	1.20	1.46	2.97

Table 3. Personal exposure to benzene (ln μg/m^3) by outdoor benzene concentration, cotinine, gender, age, season, province of residence, and caseness

A. Whole data-set (261 observation, 99 children)			
	β	**95% CI (β)**	**p(Z)**
Outdoor benzene(μg/m^3)	0.151	0.12; 0.19	<0.001
Gender (male vs female)	-0.052	-0.21; 0.11	0.522
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.027	-0.20; 0.25	0.814
[4-6) years	-0.147	-0.32; 0.03	0.098
Season	Reference Spring		
Summer	-0.027	-0.18; 0.12	0.717
Autumn	0.317	0.16; 0.48	<0.001
Winter	0.330	0.17; 0.49	<0.001
Residence	Reference = Turin		
Milan	-0.038	-0.28; 0.20	0.759
Florence - Rome	-0.208	-0.45; 0.03	0.091
Catania - Palermo - Cagliari	-0.086	-0.31; 0.13	0.443
Case vs control	-0.039	-0.19; 0.12	0.623
R^2 overall =0.4617 (within = 0.5364; between = 0.3603); Wald χ^2=234.0; p<0.0001			

B. Restricted data-set (≥2 repeats; 175 observations, 61 children)			
	β	**SE (β)**	**p(Z)**
Outdoor benzene(μg/m^3)	0.123	0.020	<0.001
Cotinine (μg/g creatinine)	0.023	0.011	0.039
Gender (male vs female)	-0.057	0.116	0.623
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.050	0.161	0.757
[4-6) years	-0.199	0.121	0.100
Season	Reference = Spring		
Summer	-0.055	0.081	0.494
Autumn	0.382	0.087	<0.001
Winter	0.351	0.086	<0.001
Residence	Reference = Turin		
Milan	0.038	0.155	0.807
Florence - Rome	-0.323	0.195	0.099
Catania - Palermo - Cagliari	-0.00001	0.138	1.000
Case vs control	-0.073	0.107	0.498
R^2 overall =0.4858 (within = 0.5564; between = 0.3544); Wald χ^2=171.89; p<0.0001			

Table 4. Urinary excretion of S-PMA (ln µg/g creatinine) by personal benzene exposure (model 1) or outdoor benzene concentration plus urinary cotinine (model 2), controlling for gender, age, season, province of residence, and caseness

Model 1 (310 observations, 98 children)	β	**95% CI (β)**	**p(Z)**
Personal benzene exposure (µg/m^3)	0.031	0.004; 0.06	0.024
Gender (male vs female)	-0.027	-0.16; 0.11	0.695
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.395	0.22; 0.57	<0.001
[4-6) years	-0.011	-0.16; 0.14	0.890
Season	Reference Spring		
Summer	0.043	-0.09; 0.17	0.514
Autumn	0.250	0.11; 0.38	<0.001
Winter	0.156	0.01; 0.30	0.033
Residence	Reference Turin		
Milan	0.007	-0.21; 0.23	0.949
Florence - Rome	0.013	-0.18; 0.21	0.898
Catania - Palermo - Cagliari	0.068	-0.14; 0.27	0.514
Case vs control	0.053	0.647	0.415
R^2 overall =0.1894 (within = 0.1263; between = 0.2174); Wald χ^2=58.97; p <0.0001			

Model 2 (214 observations, 98 children)	β	**95% CI (β)**	**p(Z)**
Outdoor benzene concentration (µg/m^3)	0.009	-0.02; 0.04	0.605
Cotinine (µg/g creatinine)	0.014	0.001; 0.03	0.040
Gender (male vs female)	-0.012	-0.16; 0.14	0.875
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.308	0.08; 0.54	0.008
[4-6) years	0.055	-0.11; 0.22	0.516
Season	Reference Spring		
Summer	-0.040	-0.18; 0.10	0.582
Autumn	0.200	0.04; 0.36	0.012
Winter	0.082	-0.07; 0.24	0.305
Residence	Reference Turin		
Milan	-0.053	-0.28; 0.18	0.657
Florence - Rome	0.048	-0.18; 0.28	0.687
Catania - Palermo - Cagliari	0.003	-0.21; 0.22	0.974
Case vs control	0.011	-0.14; 0.16	0.882
R^2 overall =0.1158 (within = 0.1423; between = 0.0925); Wald χ^2=27.59; p = 0.0063			

Exposure to benzene and childhood leukaemia: a pilot case-control study

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Word count: ~~4552~~4654

ABSTRACT

Objectives

Main purpose: to ~~assess~~ evaluate the feasibility of a measurement-based assessment of ~~personal~~ benzene exposure in case-control studies of paediatric cancer.

Additional aims: to identify the ~~main~~ sources of exposure variability ~~in personal exposure~~; to ~~evaluate~~ assess the performance of two benzene biomarkers; to verify the occurrence of participation bias; to check whether exposures to benzene and to 50 Hz magnetic fields were correlated, and might exert reciprocal confounding effects.

Design

Pilot case-control study of childhood leukaemia and exposure to benzene assessed by repeated seasonal weekly measurements in breathing zone air samples and outside the children's dwellings, with concurrent determinations of cotinine, *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) in urine.

Participants

108 cases and 194 controls were eligible for inclusion.

~~Full participation obtained from 46 cases and 60 controls, with low dropout rates before 4 repeats (11% and 17%); additional 23 cases and 80 controls allowed collection of outdoor air samples only.~~

Results

Full-participation was obtained from 46 cases and 60 controls, with low dropout rates before 4 repeats (11% and 17%); additional 23 cases and 80 controls allowed collection of outdoor air samples only.

The average benzene concentration in personal and outdoor air samples was 3 µg/m^3 (SD 1.45) and 2.7 µg/m^3 (SD 1.41), respectively.

Personal exposure was strongly influenced by outdoor benzene concentrations, higher in the cold seasons than in warm seasons, and not affected by gender, age, area of residence, or caseness.

Urinary excretion of S-PMA and personal benzene exposure were well correlated.

Outdoor benzene levels were lower among participant controls compared to non-participants, but did not differ between participant and non-participant cases; the direction of the bias was found to depend on the cut-point chosen to distinguish exposed and unexposed.

Exposures to benzene and ELF-MF were positively correlated.

Conclusions

Repeated individual measurements are needed to account for the seasonal variability in benzene exposure, and have the additional advantage of increasing the study power. Measurement-based assessment of benzene exposure in studies of ~~paediatric cancer~~childhood leukemia appear feasible, although financially and logistically demanding~~, .appear feasible and acceptable to children and their parents.~~

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Article focus

- Benzene is an established ~~causative factor for~~cause of acute non lymphocytic leukaemia (AnLL), and there is limited evidence ~~limited evidence~~ for an association between exposure to this agent and other hematologic neoplasms ~~including acute lymphocytic leukaemia~~. ~~Exposure to benzene would increase the risk of leukaemia at relatively high levels of lifetime environmental exposure (≥120 ppb). While it seems unlikely that benzene is a major cause of leukaemia in the general population, children may represent a subpopulation with increased susceptibility. Available~~ Epidemiologic studies of benzene and childhood leukemia have provided inconsistent results, possibly due to the use of surrogate exposure proxies, and lack of analyses by leukaemia subtype. ~~To get further insights on this topic, epidemiological studies based on objective estimates of environmental exposure to benzene have been recommended.~~

- Our pilot study was aimed at evaluating the logistic feasibility of an assessment of ~~personal~~ benzene exposure based on repeated ~~individual~~ measurements ~~within~~ in a case-control study of childhood leukemia. ~~.~~ A few methodological issues were also addressed (putative determinants of ~~Additional aims were: (i) to estimate the level of benzene exposure in children and assess if, and how much,~~ exposure variability; ~~was affected by a number of putative determinants; (ii) to evaluate the~~ performance of urinary levels of ~~t,t-~~muconic acid (MA~~)~~ and ~~sulpho-phenylmercapturic acid (~~S-PMA~~)~~ as benzene biomarkers in children; ~~(iii) to assess the presence of~~ participation bias ~~(which occurs when adhesion to the study protocol is associated with both the level of exposure and the presence / absence of the disease)~~; possible reciprocal confounding effects of ~~(iv) to determine whether~~ exposures to benzene and to ~~50 Hz magnetic fields (ELF-MF)~~ELF-MF) ~~were correlated, so that they could exert reciprocal confounding effects in the analyses of their relationship with childhood leukemia~~.

Key messages

- ~~Eligibility~~ Eligible for inclusion ~~was restricted to~~were 108 cases and 194 matched controls, aged 2 to 12 years at the time of the survey. Full participation rates were low, ~~(cases 43%, controls 31%)~~, but the outdoor monitoring was accepted by ~~additional 21~~64% of cases and ~~41~~72% of controls ~~accepted the outdoor monitoring~~. Adherence of full participants to the scheduled ~~four seasonal~~ repeats was very satisfactory (cases 89%, controls 83%).

- Personal exposure was strongly influenced by outdoor benzene concentrations, was higher in the cold seasons than in warm seasons, and was not affected by gender, age, area of residence, or caseness. Personal benzene exposure and urinary excretion of S-PMA (but not of MA) were well correlated. ~~Outdoor benzene levels were lower among participant controls compared to non-participants, but did not differ between participant and non-participant cases (a~~A participation bias was indeed present~~)~~. A positive association between exposures to benzene and ELF-MF was observed.
- Epidemiologic studies of paediatric cancer and estimates of environmental benzene exposure based on repeated seasonal measurements, although challenging, appear logistically feasible ~~and acceptable to children and their parents~~.

Strengths and limitations

- To our knowledge, this is the first pilot study of childhood leukaemia and measured personal benzene exposure. ~~Its also has the merit of having addressed a number of methodological problems besides logistic feasibility issues.~~
- ~~Due to logistic reasons and resource constraints, t~~The study size ~~was~~ is very small. ~~It must also be stressed that the~~ The ~~expected~~ greater accuracy of measurement-based exposures estimates, compared to surrogate exposure proxies, does not necessarily correspond to increased ~~construct~~ validity~~; this is,~~ especially ~~true~~ when measurements are used for retrospective post-diagnosis exposure assessments.

INTRODUCTION

Benzene is a ubiquitous air pollutant, that needs to be metabolized to become carcinogenic.[1- 2]

Benzene exposure and acute non lymphocytic leukaemia (AnLL) are causally related in adult humans, while there is limited evidence for an association between exposure to this agent and acute or chronic lymphocytic leukaemia, multiple myeloma, and non-Hodgkin's lymphoma.[3] Moreover, a dose-dependent association between benzene exposure and incidence of myelodysplastic syndrome has been observed among petroleum workers. [4]

Exposure to benzene would increase the risk of AnLL ~~leukaemia~~ at levels of ≥40 ppm-years of occupational cumulative exposure, equivalent to a lifetime (76 years) environmental exposure of ≥120 ppb.[45]

Due to the established carcinogenicity of benzene, WHO has not developed any guideline value for this chemical in air, while indicating that ambient benzene concentrations of 17, 1.7 and 0.17 $\mu g/m^3$ are associated with excess lifetime risks of leukaemia of 10^{-4}, 10^{-5} and 10^{-6}, respectively.[~~5~~6-~~6~~7]

While it seems unlikely that benzene is a major cause of leukaemia in the general population exposed in the ppb range, children may represent a subpopulation with increased susceptibility ~~on intake or on key pharmacokinetic / pharmacodynamic processes~~.[1, 3]

Childhood leukaemias have distinctive features compared to leukaemias in adults. The major subtypes are acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML), accounting for 80% and 15% of cases aged 0 to 14 years in white populations respectively.[8] Both subtypes are thought to develop through a first initiating event *in utero* (e.g. the TEL-AML1 gene fusion whose prevalence in newborns has been estimated at around 1% while it is observed in 25% of ALL cases) followed by further postnatal genetic changes.[8] The "second hit" might consist of

additional idiopathic chromosomal translocations, as well as of exposures to biological, chemical or physical agents~~In precursor B-cell acute lymphoblastic leukaemia (pre-B ALL) and some cases of acute myeloid leukaemia (AML), a first initiating genetic event has been shown to occur *in utero*, at a rate of up to 1% (for TEL-AML1 translocations in pre-B ALL). Further genetic changes are required to create a malignant clone~~.[9] Ionizing radiation, benzene, alkylators and topoisomerase II inhibitors are among the few confirmed environmental risk factors for AML, while delayed, dysregulated responses to common infections are likely to play a major role in the conversion of pre-leukemic clones into overt ALL.[~~7~~8-9]

Findings from available studies of benzene and childhood leukaemia are inconsistent, possibly due to the use of indirect estimates of exposure and lack of analyses by leukaemia subtype.[~~8~~10]

To advance current understanding of benzene health effects and susceptibility, studies of paediatric cancers that include estimates of environmental exposure to benzene, rather than surrogate exposure indicators, have been recommended.[~~9~~11]

Major challenges in pursuing this suggestion include the space- and time-variability of ambient benzene levels, the low exposure levels in children, and the inherent susceptibility of case-control studies (the design of choice for etiological studies of rare disease like childhood cancer) to selection and information bias.

We evaluated the logistic feasibility of an assessment of benzene exposure based on repeated seasonal weekly measurements in breathing zone air samples and outside the children's dwellings, with concurrent determinations of cotinine, *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) in urine, in a pilot investigation within an Italian case-control study on environmental risk factors for childhood leukaemia (SETIL).

Additional objectives of the pilot study were:

- to investigate the relationship between level personal exposure to benzene and putative determinants (atmospheric benzene, second-hand tobacco smoke, individual traits);
- to assess the performance of *t*-*t*-muconic acid (MA), and sulpho-phenylmercapturic acid (S-PMA) as benzene biomarkers in children;
- to verify the occurrence of participation bias from differential adhesion to the benzene measurement study, and estimate the amount and direction of the distortion;
- to check whether exposures to benzene and to extremely low frequency magnetic fields (ELF-MF) were correlated, and might eventually exert reciprocal confounding effects on the relationship with childhood leukaemia.

METHODS

Study population

Incident cases of childhood leukaemia from 14 Italian regions, aged 0 to 10 years at diagnosis in 1998-2001, were eligible for enrolment in the SETIL study. Cases were ascertained through the national registry run by the Association of Paediatric Haematology and Oncology (AIEOP). Controls, matched to cases (2:1 ratio) on gender, date of birth, and region, were randomly selected from population lists. Information on several items concerning the children, their next-of-kin and dwellings, was collected by interview of parents. All interviewed families were invited to participate in a measurement study of indoor ELF-MF, while subsets of participants were asked to join two side-investigations, on exposure to gamma radiation and benzene, respectively.

Eligibility for the benzene pilot study was restricted to 108 childhood leukaemia cases from seven Italian provinces (Turin, Milan, Florence, Rome, Catania, Palermo, and Cagliari), diagnosed between July 2000 and December 2001, and 194 matched controls.

The study protocol was approved by the Piedmont Ethical Committee on 14 January 2002.

Sampling strategy and devices

Due to the high daily and seasonal variability of atmospheric benzene concentrations, the protocol called for four repeated seasonal one-week samplings of breathing zone air per child over one year ("personal" air samples), with concurrent collection of urine samples and atmospheric air samples in proximity of the children's homes ("outdoor" air samples).

Outdoor air sampling would also be performed, with an identical strategy, near the homes of all eligible non-participants.

To study the day-to-day variability in exposure, 24-h repeated personal and indoor samples during four season-specific weeks would be collected from a subset of children and related homes.

Personal air samples were collected by passive samplers (Radiello® radial symmetry diffusive sampler) worn by the child during the day and placed at the bedside at night.

Radiello® samplers were also used to collect outdoor air samples, placed near the entrance of the dwellings (within 1 meter), at a vertical distance from the ground suitable to avoid infringements (2-2.5 m), stored in a plastic case to avoid rain or snow.

At retrieval, the adsorbing cartridges were removed from the diffusive bodies and placed into glass storage tubes. The ID code of the child, along with dates and times of sampling start and end, were recorded on self-adhesive labels stuck on the tubes. The cartridges were sent to a single laboratory (Fondazione Salvatore Maugeri, Padova) for the chemical analyses.

Daily urine samples (10 ml, from the last micturition before sleep) were collected for 7 subsequent days (70 ml per week) during each seasonal survey. The daily samples were pooled in one plastic vial, and kept in the freezer compartment of the home refrigerator until collection at the end of the week. The vials were transported to the local research centre in cool bags, and stored at –5 °C

until delivery (packed in dry ice and usually in 2 weeks) to the laboratory (Fondazione Salvatore Maugeri, Pavia).

Field work began between March 2002 and January 2003, and ended in October 2003 - July 2004, depending on the local research centre.

Chemical determinations

Benzene concentrations in air sample were determined by an automated thermal desorber (ATD400, Perkin Elmer) coupled to a capillary gas-chromatography system (Autosystem XL, Perkin Elmer). The expanded uncertainty of the method, in the range 2.4 to 14.3 µg/m^3, was shown to be 18%.[~~10~~12] The limits of detection and quantification, over 1 week exposure, are 0.05 µg/m^3 and 0.1 µg/m^3.

The urine analyses were performed using a high pressure liquid chromatography system (Alliance 2690, Waters) equipped with a spectrometric (SM) detector (ZQ, Waters) following a preliminary step of purification of the samples on pre-activated solid phase extraction (SPE) cartridges. The limit of detection (LOD), coefficient of variation (CV) and accuracy of the method were: LOD = 1 µg/L, CV % = (1.22)-(1.10), accuracy % = (- 2.39)-(3.36) for S-PMA; LOD = 20 µg/L, CV % = (1.33)-(1.06), accuracy % = (- 2.18)-(3.27) for MA; LOD = 1 µg/L, CV % = (1.25)-(1.09), accuracy % = (-2.29)-(3.33) for cotinine.

Further details are provided in Appendix 1.

The chemical determinations were completed by May 2005.

Statistical analyses

Measurements below the chemical-specific detection limits were assigned half such values and included in the analyses.

The relationships between personal exposure to benzene and putative determinants (as well as between urinary excretion of benzene metabolites, benzene intake, and other covariates) were assessed by generalized least squares (GLS) models for repeated measurements (STATA v. 11, xtreg procedure). The GLS model is: $y_{it} = \alpha + X_{it}B + u_{it} + e_{it}$, where i (1 to n) is the number of observations collected at time t (1 to 4) and u_{it} and e_{it} are the error components.

As concentrations of benzene and urinary analytes were log-normally distributed, we always included in the models log-transformed dependent variables.

We used the odds ratio (OR), calculated from generalized estimating equations (GEE) for repeated individual measurements (STATA v. 11, procedure xtgee), to estimate the association between benzene exposure and dichotomous variables such as case-control or participation status. The general equation of the GEE model is $g\{E(y_j)\}=x_j\beta$, where g is the link function, herein a logit function.

We calculated a participation bias factor following the method suggested by Greenland [bias factor = $(S_{1a}*S_{0b})/(S_{0a}*S_{1b})$], where S_{1a}, S_{0a}, S_{1b}, and S_{0b} denote the probabilities of selection (i.e. full participation in the benzene study) for exposed cases, unexposed cases, exposed controls, and unexposed controls.[~~11~~13] When the bias factor equals 1, there is no bias, when it is above or below 1 the true OR will be biased respectively upward or downward by the magnitude of this factor.

Multiple regression models were used to analyze the relation between estimated exposures to benzene and ELF-MF.

RESULTS

Participation and sampling outcome

Out of 108 cases and 194 controls eligible for inclusion, 46 cases and 60 controls (43% and 31%) agreed to take full part in the benzene side-study (Figure 1).

In addition, the parents of 23 cases and 80 controls who refused the personal exposure assessment accepted the outdoor monitoring (partial participation = 21% and 41%).

Altogether 1467 air samples were collected. A small percentage (2%) were lost during monitoring (22 samplers stolen, 2 sampler plates broken, 3 cartridges lost), transport (8 missing labels) or chemical analysis (2 cartridges broken on arrival at the laboratory; 1 sample lost due to equipment failure).

Benzene measurements from the day-to-day variability sub-study (19% of the total) could not be used because only four control children accepted the 24-h sampling scheme, and were replaced by the calculated weekly averages.

A further 20% of benzene measurements was removed from the data-set due to lack of compliance with the study protocol (indoor samples collected in place of the personal ones from children refusing to wear the sampler; time-or place-mismatch of personal and outdoor samples; "orphan" personal or outdoor samples; duplicate season-specific measurements; non-participants replaced with children ineligible for the benzene side-study].

For the same reasons, 107 out of 417 chemical determinations in urine (26%) were discarded.

Three cases and 5 controls were excluded from one or more analyses due to lack of complete measurement sets in all seasonal series and, although 89% and 83% of full-participant cases and controls did adhere to all four seasonal surveys, only 37% and 43% of them had four repeated analyzable observations.

Personal characteristics of the children

The families of cases participating in full to the benzene study had been interviewed on average 1.3 years (SD 0.47) after the date of diagnosis, and the control-families 1.5 years (SD 0.46) after the corresponding reference date. The delay between diagnosis and the first series of benzene measurements was 2 years (SD 0.53) for both cases and controls.

Cases and controls were comparable in terms of gender, age, and father's attained educational level (Table 1). A higher proportion of controls than cases had both parents smoking, and control-mothers were more educated than case-mothers. There were similar proportions of only children in the case and control groups, while firstborn children were more frequent among controls than cases. Early schooling (day-care attendance ~~of crèche~~) was more common in cases than in controls. At the time of the benzene survey, most children were still living in the home occupied at birth or in the house they moved into after birth but before the date of diagnosis (cases 95%; controls 91%).

Level, variability, and determinants of personal exposure to benzene

The analyses of level, variability and determinants of personal exposure to benzene were based on 43 cases (39 ALL and 4 AML) and 56 controls, with 261 valid pairs of benzene concentrations in breathing zone and outdoor air (110 from cases and 151 from controls). A large proportion of these children (35%) had a single pair of concurrent measurements, unevenly distributed by season, with a disproportionally high number of summer samples (30 out of 35, all but one from a single centre).

The distributions, overall and by season, of benzene concentrations in personal and outdoor air samples, and of cotinine, MA and S-PMA in urine are described in Table 2.

Personal exposure to benzene was log-normally distributed (Shapiro-Wilk test = 0.938, $p<0.001$), and the mean benzene level over the individual yearly averages was 3 $\mu g/m^3$ (0.92 ppb).

The distribution of benzene outdoor concentration was skewed to the left in all seasons and the yearly averages were log-normally distributed as well (Shapiro-Wilk test = 0.948, p = 0.001); the average yearly benzene level near the children's homes was 2.7 $\mu g/m^3$ (0.83 ppb).

Both outdoor benzene concentrations and personal exposure levels were higher in the cold seasons (autumn-winter) than in the warm ones (spring-summer).

The European limit for benzene in air (5 $\mu g/m^3$) was exceeded by 5% of the yearly average outdoor concentrations, and by 8% of the yearly average levels in breathing zone air samples. A large proportion of autumn and winter measurements were above 5 $\mu g/m^3$ (35% and 25% outdoor; 26% and 30% of the personal exposure estimates).

Cases and controls had similar levels of personal exposure to benzene: the leukaemia OR for a unit increase (1 $\mu g/m^3$) in personal benzene exposure was 0.93 (95% CI 0.77-1.13) adjusting for gender, age at the benzene survey (2-4; 4-6; 6-12 years), cotinine in urine (µg/g creatinine), season, and province of residence (Turin; Milan; Florence - Rome; Catania - Palermo - Cagliari).

A similar lack of association was found between the odd of disease and benzene concentration outside the children's homes [OR 0.94 (95% CI 0.80-1.09)], controlling for gender, age, smoking habits of the parents at the interview (non-smokers, mother or father smoking; both parents smoking), season, and province of residence.

Further adjustment for birth order and age at first schooling had no material effect on the observed leukaemia-benzene relationship [personal exposure: OR 0.92 (95% CI 0.75-1.13); outdoor benzene: OR 0.95 (95% CI 0.81-1.13)].

As cases and controls had comparable levels of benzene exposure, we carried out the analyses illustrated in the forthcoming paragraphs on the whole data-set, although always controlling for caseness.

Urinary cotinine concentration (μg/g of creatinine) was higher in children of smoking parents compared to children of non-smokers, and children with both parents smoking excreted a larger amount of cotinine than children with one parent smoking (Appendix Table A). Cotinine levels were higher in winter than in other seasons, and higher in children from central and southern Italy (Florence, Rome, Palermo, Catania, Cagliari) than in children from northern provinces (Turin and Milan). The high between- *vs* within-subject R^2 ratio is worth noting.

Personal benzene exposure was strongly influenced by outdoor benzene concentrations (Table 3-A), and apparently not affected by gender or age; the season showed a modifying effect, with increasing levels of personal exposure during autumn and winter; the fraction of variability explained by the model was higher for the within-subject component than for the between-subject one.

Exposure to second-hand tobacco smoke (estimated by cotinine excretion or by parental smoking habits) showed a trivial influence on personal exposure to benzene. The inclusion of urinary cotinine (μg/g creatinine) in the model described in Table 3-A, slightly decreased its goodness of fit [R^2 overall = 0.46; Wald χ^2=189.49; R^2 within = 0.55; R^2 between = 0.35; β (cotinine) = 0.012; 95% CI = -0.003; 0.03)]; an alternative model, including smoking habits of the parents, did not perform any better [R^2 overall = 0.46; Wald χ^2=216.44; R^2 within = 0.52; R^2 between = 0.39; β (one parent smoking) = 0.14; 95% CI = -0.02; 0.31; β (both parents smoking) = 0.17; 95% CI = -0.06; 0.39].

Children from central Italy (Florence and Rome) tended to have lower benzene concentrations in breathing zone air samples compared to residents in other provinces, all other things being equal (Table 3-A), possibly because of residual confounding from lack of samples collected in Rome other than in summer. We tried to verify this hypothesis by restricting the analyses to children with at least two series of measurements in different seasonal periods (cold and warm). The data-set reduced to 61 subjects (25 cases and 36 controls) and 220 pairs of personal-outdoor benzene

measurements. Actually, children from Florence still showed (not significantly) lower levels of personal exposure to benzene (β = - 0.27; 95% CI = -0.56; 0.03; p =0.074) compared to children from Turin. In the restricted data-set, however, independent effects of both outdoor benzene and urinary cotinine levels on personal benzene exposure were observed (Table 3-B).

Benzene intake and urinary excretion of benzene metabolites

Ninety-eight children (43 cases and 55 controls) and 310 pairs of urine and breathing zone air measurements (138 from cases and 172 from controls) were available for the analyses of the urinary excretion of benzene metabolites (MA and S-PMA) in relation to personal exposure to benzene.

Urinary concentrations of S-PMA (ln µg/g creatinine) were related to personal exposure to benzene (Table 4, Model 1). Youngest children (2-4 years at the benzene survey) excreted higher level of S-PMA compared to children aged 6-12 years, all other conditions being equal, and urinary concentration of S-PMA were higher in samples collected during the cold seasons compared to spring samples. The model, however, explained just 19% of the overall S-PMA variability. In an alternative model, including outdoor benzene concentrations and urinary cotinine in place of personal benzene exposure, we also observed an effect of the nicotine biomarker on S-PMA excretion (Table 4, Model 2).

On the contrary, neither benzene concentrations in breathing zone air samples, nor outdoor benzene concentrations or cotinine levels explained the intra- and inter-individual variability in urinary levels of MA, controlling for gender, age, season, area of residence, and caseness (data not shown).

Bias due to differential participation

Available for the analysis of participation bias were 66 cases (43 full-participant and 23 partial-participant) and 136 controls (56 and 80), with 652 measurements of outdoor benzene concentrations (135 and 175 from full-participant cases and controls; 81 and 261 from partial-participant cases and controls).

Benzene concentrations near the homes of full-participant controls were significantly lower than those in proximity of partial-participants' dwellings (OR = 0.88; 95% CI 0.80-0.97), adjusting for gender, age, season and place of residence, while there was no difference in ambient benzene levels between participant and non-participant cases (OR = 0.95; 95% CI 0.82-1.09). As participation in the study was also associated with the case-control status, assuming a causal association between exposure and disease, a selection bias might ensue. However, as parents of more exposed controls were less willing to accept to be interviewed, an upward distortion would be expected, which is at odds with the apparent lack of association between personal benzene exposure and leukaemia risk in the current study.

To the aim of the current analysis, personal exposure to benzene was dichotomized around the median (3.25 $\mu g/m^3$), the 75th percentile (4.34 $\mu g/m^3$) or 5 $\mu g/m^3$ (the current limit for airborne benzene in Europe). The amount and direction of bias were found to depend on the cut-point chosen (Appendix Table B), whereas no bias is expected when the exposure is categorized around the median (bias factor = 1.03), and biases in the opposite directions are predicted using cut-off at p75 and at 5 $\mu g/m^3$ (0.64 and 1.42, respectively).

Relationship between exposures to benzene and ELF-MF

Children with benzene and ELF-MF measurements made at the same house qualified for inclusion in the analysis of the relationship between estimated exposures to these agents. As only 35 cases and 46 controls met such criterion when benzene concentrations in breathing zone air samples were used as exposure indicator, we performed the analysis on 48 cases and 77 controls with

place-comparable pairs of average yearly outdoor benzene concentration (μg/m^3) and 48 h TWAs of ELF-MF level in the child's bedroom (ln μT).

There was a positive association between estimated exposures to ELF-MF (dependent variable) and benzene (β = 0.177; 95% CI 0.06-0.29; p = 0.002); the multivariable regression model (including gender, age, province of residence, caseness, and participation in the benzene pilot study as covariates) explained 16% of the variability in the dependent variable [F (10, 114 df) = 2.13; p> F = 0.0271]. A steeper increase in ELF-MF level per unit increase in outdoor benzene concentration (β = 0.520; 95% CI 0.09-0.95; p = 0.019) was seen among the 81 children fully participating in the benzene pilot-study compared to the 44 partial-participants (Appendix Table C).

Similar results, with a more accentuated increase in indoor magnetic induction level per unit increase in outdoor benzene concentration [β= 0.272; 95% CI = 0.09-0.45; p(t) = 0.003; R^2 = 0.19], were observed in the restricted data-set of 86 children with ≥2 weekly samplings in alternate seasons.

DISCUSSION

We have carried out a pilot case-control study of childhood leukaemia and exposure to benzene assessed by repeated individual measurements made on average two years after diagnosis. The pilot study included side-investigations aimed at evaluating the performance of two biological indicators of benzene exposure in children, at estimating amount and direction of a possible participation bias, and at assessing the relation between estimated exposures to benzene and ELF magnetic fields.

Due to the relatively low incidence of childhood cancers (10-15 for 100,000 person-years in the 0-14 year range in most industrialized countries), the case-control approach is the design of choice

for analytical epidemiologic studies about potential risk factors for these diseases. Such a study design, however, is inherently prone to measurement errors stemming from the retrospective reconstruction of the exposures of interest, and to differential participation leading to control samples not representative of the study base. Therefore, findings from observational epidemiologic studies of postulated determinants for childhood malignancies are often inconsistent and always require a cautious and thoughtful interpretation.[~~12~~14]

Although based on small numbers, some of the findings from the current study have a certain factual and methodological interest.

Repeated samplings of breathing and outdoor air are indeed needed to account for the seasonal variability in environmental benzene levels.[~~13~~15-~~14~~16]

On average, children participating in the current study appear to experience mean yearly levels of personal exposure to benzene not exceeding the European guidelines (although 8% percent of the yearly mean levels were above 5 µg/m^3).

What we *a priori* considered the main sources of benzene exposure for children (ambient benzene levels and second-hand tobacco smoke) explained no more than half of the overall variability in personal exposure, which indicates the need to identify other sources of exposure particularly relevant, perhaps, during the cold seasons. In fact, in autumn-winter compared to spring-summer, we observed higher levels of personal exposure to benzene, of urinary cotinine and of S-PMA excretion, all other things being equal. These findings might be due to the lower ventilation rates in homes and schools during the cold seasons, to winter-specific sources of indoor benzene concentrations not considered in the current survey (e.g. fireplaces or other combustion sources), and/or to the seasonal variability in daily patterns of time spent in different micro-environments (e.g. within cars or buses).[~~15~~17]

Some case-control studies have suggested an association between exposure to traffic density and childhood leukaemia;[~~16~~18-~~19~~21] however, negative findings have also been reported.[~~20~~22-~~23~~25] Positive associations between incidence of ALL in children and residential proximity to petrol stations were observed in three case–control studies.[~~21~~23, ~~24~~26-~~25~~27] An increased risk of childhood leukaemia in relation to estimated exposure to benzene was observed in a small Italian study,[~~26~~28] but not in a much larger case-control study carried out in Denmark and based on a sophisticated and validated exposure modelling.[~~27~~29]

To our knowledge there is no previous study of childhood leukaemia and measured personal benzene exposure. Moreover, as only children aged 0 to 10 years at diagnosis were eligible for the SETIL study, the large majority of cases included in the current investigation were pre-B ALL.

Cases and controls did not differ in terms of exposure to benzene, estimated either by benzene level in personal air samples or through outdoor benzene concentration, but the interpretation of this finding is hampered by the retrospective exposure assessment and the low statistical power of this preliminary investigation. That notwithstanding, due to the design based on repeated individual observations, the risk estimates have quite narrow confidence intervals. Thus the findings from this pilot study, in accordance with the limited evidence for an association between exposure to benzene and ALL,[3~~-~~, 45] might also suggest that the levels of benzene exposure experienced by children living in Italian towns do not entail a detectable increase in the risk of ALL.

Current perspectives on the causes of childhood ~~leukaemia~~ ALL increasingly point towards an etiologic role of altered patterns of infections and related immune stimulation during the first years of life, and one piece of supporting evidence is the consistent observation of an inverse association between ALL risk and day-care attendance.[~~28~~30] Studies of childhood ALL and birth order, on the other hand, have provided inconsistent result.[~~29~~31] Neither age at first schooling,

nor birth order confounded the relation between childhood leukaemia and indicators of benzene exposure in the current study.

S-PMA concentration measured in repeated weekly samples of the last micturition before sleep was found to reflect personal exposure to benzene, although the available covariates explained a small fraction of the within- and between-subject variability of this benzene metabolite. This is a quite surprising result, considering that S-PMA is believed to represent less than 1% of urinary benzene metabolites for exposures to benzene at air concentrations between 0.1 and 10 ppm.[~~30~~32]

Benzene exposure proved not able to explain the variability of MA urinary excretion observed in our children, consistent with findings from a previous Italian study.[~~31~~33] The low statistical power of the study, the low level of benzene exposure, and the lack of adjustment for the confounding effect of dietary intake of sorbic acid (a common food additive), may explain this finding.[~~32~~34]

Full-participation rates were low, in line with a general tendency to decreasing participation rates, especially in epidemiological studies requiring adherence to complex measurement protocols.[14, 35] ~~higher among cases than controls.~~ That n~~N~~otwithstanding, the~~–~~ outdoor monitoring was accepted by a fairly satisfactory proportions of ~~children~~ families ~~with measured outdoor benzene concentrations~~ (~~61~~64% and ~~70~~72% of eligible cases and controls)~~, the degree of partial participation was lower among non participant cases (21%) than among non participant controls (41%)~~ This is an encouraging result, given the strong correlation between personal benzene exposure and ambient benzene level observed in the current study.

We observed a differential participation bias, which underscores the need to plan parallel bias analyses in any case-control study.[~~33~~36] The dependence of the participation bias factor on the cut-point chosen to dichotomize the exposure variable is of methodological interest.

The positive association between the 48 h TWA of ELF-MF induction in the child's bedroom and the average yearly concentrations of outdoor benzene will need consideration in the interpretation of findings from the analyses of childhood leukaemia risk in relation to 50 Hz MF in the SETIL case-control study.

Incidental failures during sample collection, transport or chemical analysis accounted for a negligible proportion of lost air or urine samples. However, substantial percentages of chemical measurements could not be included in current analyses because of misunderstanding of the sampling protocol.

The day-to-day variability sub-study was clearly too demanding to be acceptable.

In conclusion, the current pilot study suggests that epidemiologic studies of childhood leukaemia risk and measurement-based estimates of exposure to benzene are challenging but logistically feasible (provided that the study protocol specifies every single sampling detail and nothing is considered so obvious as to be omitted). Such an exposure assessment method could be considered by epidemiologists willing to involve in the "genome - exposome" approach to gain further insight into the relationship between benzene exposure and childhood leukaemia risk, with priority given to AML.[~~4~~2, ~~34~~37-39] Due to the low incidence rates of AML in children, however, international multi-centre studies are needed to address this topic.

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COMPETING INTERESTS

None.

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Table 1. Children included in the pilot study by selected characteristics

		Cases		Controls	
		N	%	N	%
Gender	Female	25	58	30	54
	Male	18	42	26	46
Age at the survey	[2,4) years	5	12	9	16
	[4,6) years	21	49	16	29
	[6,12] years	17	40	31	55
Residence*	Turin	7	16	9	16
	Milan	8	19	13	23
	Florence	3	7	5	9
	Rome	14	33	15	27
	Catania	3	7	5	9
	Palermo	4	9	6	11
	Cagliari	4	9	3	5
Parent smoking§	None	20	47	27	48
	One	16	37	18	32
	Both	4	9	11	20
	Missing	3	7	0	-
Father's education§	No qualification	-	-	1	2
	Primary school	17	40	21	38
	High school	17	40	24	43
	University degree	6	14	10	18
	Missing	3	7	-	-
Mother's education§	No qualification	-	-	-	-
	Primary school	19	44	17	30
	High school	15	35	26	46
	University degree	9	21	13	23
	Missing	-	-	-	-
Birth order§	Only child	10	23	12	21
	First born	10	23	20	36
	Second born or higher birth order	23	53	24	43
Age at first schooling§	No schooling yet	15	35	16	29
	<3 years (crèche)	14	33	9	16
	[3,6) years (preschool)	14	33	30	54
	[6-7] years (primary school)	0	-	1	2
Home at the time of the benzene survey^	Occupied since birth	28	65	39	70
	Moved into after birth & before diagnosis	13	30	12	21
	Moved into after diagnosis & before interview	1	2	5	9
	Moved into after interview	1	2	-	-
Total		43	100	56	100

*At the time of diagnosis or the corresponding reference date for controls; §Information reported at the interview; ^The ELF magnetic fields measurements, if the parents agreed, were made at the time of the interview.

Table 2. Benzene concentration in personal and outdoor air samples, and urine level of cotinine and benzene metabolites by season and overall

	Obs (#)	Mean	SD	G-mean	G-SD	Min	Percentiles			Max
Benzene in personal air samples (μg/m^3)							**p25**	**p50**	**p75**	
Spring	57	2.51	1.89	2.10	1.75	0.60	1.50	1.82	3.11	11.12
Summer	86	2.26	1.45	1.90	1.82	0.47	1.25	1.85	3.10	8.13
Autumn	62	4.31	2.60	3.73	1.57	0.92	2.939	3.70	5.17	18.47
Winter	56	4.04	1.78	3.67	1.73	1.55	2.34	4.00	5.24	9.03
Individual yearly averages	99	3.00	1.45	2.66	1.67	0.75	2.05	2.90	3.83	9.00
Benzene in outdoor air samples (μg/m^3)										
Spring	57	2.29	1.30	1.93	1.84	0.48	1.20	1.91	3.15	5.67
Summer	86	1.94	1.20	1.65	1.75	0.39	1.12	1.58	2.28	6.92
Autumn	62	3.99	2.58	3.05	1.92	0.08	1.93	3.42	5.63	11.18
Winter	56	3.80	1.86	3.25	2.35	0.15	2.40	3.66	5.20	8.31
Individual yearly averages	99	2.70	1.41	2.33	1.78	0.27	1.59	2.37	3.63	6.92
Cotinine (μg/ g creatinine)										
Spring	78	3.92	7.04	1.91	3.26	0.05	1.00	1.94	3.50	49.0
Summer	78	3.20	5.52	1.50	3.59	0.09	0.82	1.68	3.71	41.4
Autumn	76	4.54	8.51	1.92	3.92	0.05	1.20	1.93	4.30	48.7
Winter	74	4.36	7.38	2.32	3.01	0.10	1.20	2.30	4.80	53.5
Individual yearly averages	98	3.73	5.99	2.14	2.67	0.30	1.08	2.09	3.58	41.9
MA (μg/g creatinine)										
Spring	81	104.22	69.28	87.43	1.79	17.00	60.27	82.00	126.99	349.00
Summer	79	140.40	226.73	92.30	2.16	13.33	56.54	83.00	131.76	1680.00
Autumn	76	128.24	124.04	99.57	1.94	30.21	60.16	102.48	147.21	893.04
Winter	74	119.09	100.15	95.30	1.86	26.00	65.00	86.00	129.00	591.00
Individual yearly averages	98	116.65	84.89	101.06	1.62	46.42	73.33	92.66	122.50	593.42
S-PMA (μg/g creatinine)										
Spring	81	1.13	0.60	1.00	1.62	0.21	0.80	1.00	1.30	3.70
Summer	79	1.12	0.54	1.02	1.54	0.41	0.72	1.00	1.39	3.30
Autumn	76	1.53	0.93	1.33	1.67	0.49	0.97	1.29	1.84	5.80
Winter	74	1.37	0.60	1.23	1.64	0.15	1.00	1.20	1.60	3.40
Individual yearly averages	98	1.28	0.50	1.20	1.43	0.56	0.94	1.20	1.46	2.97

Table 3. Personal exposure to benzene (ln μg/m^3) by outdoor benzene concentration, cotinine, gender, age, season, province of residence, and caseness

A. Whole data-set (261 observation, 99 children)			
	β	**95% CI (β)**	**p(Z)**
Outdoor benzene(μg/m^3)	0.151	0.12; 0.19	<0.001
Gender (male vs female)	-0.052	-0.21; 0.11	0.522
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.027	-0.20; 0.25	0.814
[4-6) years	-0.147	-0.32; 0.03	0.098
Season	Reference Spring		
Summer	-0.027	-0.18; 0.12	0.717
Autumn	0.317	0.16; 0.48	<0.001
Winter	0.330	0.17; 0.49	<0.001
Residence	Reference = Turin		
Milan	-0.038	-0.28; 0.20	0.759
Florence - Rome	-0.208	-0.45; 0.03	0.091
Catania - Palermo - Cagliari	-0.086	-0.31; 0.13	0.443
Case vs control	-0.039	-0.19; 0.12	0.623
R^2 overall =0.4617 (within = 0.5364; between = 0.3603); Wald χ^2=234.0; p<0.0001			

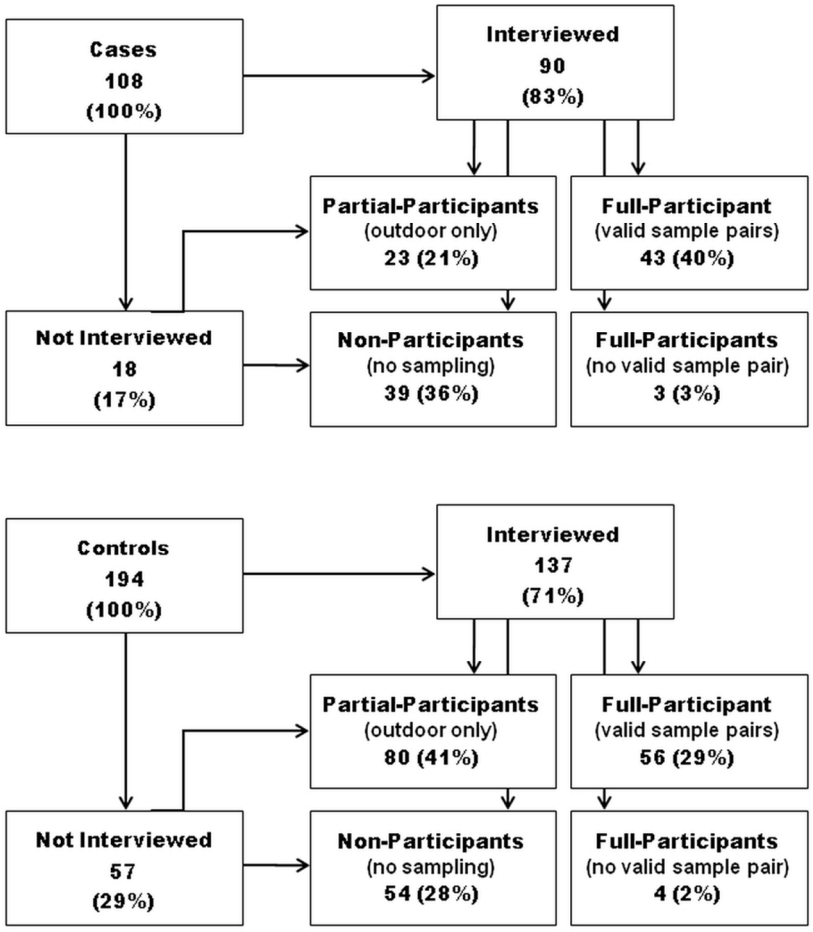
B. Restricted data-set (≥2 repeats; 175 observations, 61 children)			
	β	**SE (β)**	**p(Z)**
Outdoor benzene(μg/m^3)	0.123	0.020	<0.001
Cotinine (μg/g creatinine)	0.023	0.011	0.039
Gender (male vs female)	-0.057	0.116	0.623
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.050	0.161	0.757
[4-6) years	-0.199	0.121	0.100
Season	Reference = Spring		
Summer	-0.055	0.081	0.494
Autumn	0.382	0.087	<0.001
Winter	0.351	0.086	<0.001
Residence	Reference = Turin		
Milan	0.038	0.155	0.807
Florence - Rome	-0.323	0.195	0.099
Catania - Palermo - Cagliari	-0.00001	0.138	1.000
Case vs control	-0.073	0.107	0.498
R^2 overall =0.4858 (within = 0.5564; between = 0.3544); Wald χ^2=171.89; p<0.0001			

Table 4. Urinary excretion of S-PMA (ln µg/g creatinine) by personal benzene exposure (model 1) or outdoor benzene concentration plus urinary cotinine (model 2), controlling for gender, age, season, province of residence, and caseness

Model 1 (310 observations, 98 children)	**β**	**95% CI (β)**	**p(Z)**
Personal benzene exposure (µg/m^3)	0.031	0.004; 0.06	0.024
Gender (male vs female)	-0.027	-0.16; 0.11	0.695
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.395	0.22; 0.57	<0.001
[4-6) years	-0.011	-0.16; 0.14	0.890
Season	Reference Spring		
Summer	0.043	-0.09; 0.17	0.514
Autumn	0.250	0.11; 0.38	<0.001
Winter	0.156	0.01; 0.30	0.033
Residence	Reference Turin		
Milan	0.007	-0.21; 0.23	0.949
Florence - Rome	0.013	-0.18; 0.21	0.898
Catania - Palermo - Cagliari	0.068	-0.14; 0.27	0.514
Case vs control	0.053	0.647	0.415
R^2 overall =0.1894 (within = 0.1263; between = 0.2174); Wald χ^2=58.97; p <0.0001			

Model 2 (214 observations, 98 children)	**β**	**95% CI (β)**	**p(Z)**
Outdoor benzene concentration (µg/m^3)	0.009	-0.02; 0.04	0.605
Cotinine (µg/g creatinine)	0.014	0.001; 0.03	0.040
Gender (male vs female)	-0.012	-0.16; 0.14	0.875
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.308	0.08; 0.54	0.008
[4-6) years	0.055	-0.11; 0.22	0.516
Season	Reference Spring		
Summer	-0.040	-0.18; 0.10	0.582
Autumn	0.200	0.04; 0.36	0.012
Winter	0.082	-0.07; 0.24	0.305
Residence	Reference Turin		
Milan	-0.053	-0.28; 0.18	0.657
Florence - Rome	0.048	-0.18; 0.28	0.687
Catania - Palermo - Cagliari	0.003	-0.21; 0.22	0.974
Case vs control	0.011	-0.14; 0.16	0.882
R^2 overall =0.1158 (within = 0.1423; between = 0.0925); Wald χ^2=27.59; p = 0.0063			

Figure 1. Children eligible for inclusion and participation rates



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Appendix 1 – Chemical determination: analytical conditions

Benzene concentrations in air samples

The main analytical conditions were the following: desorption at 320 °C for 10 min; overall split ratio 1:75; carrier gas nitrogen at 27 psi; column J&W PONA, 50 m, 0.2 mm id, 0.5 µm film thickness; oven 35 °C for 1 min, 6 °C/min to 110 °C, 20 °C/min to 220 °C, 2 min.

Urine analyses

Pre-treatment and chromatographic conditions used for each analyte are described below.

S-PMA. Pre-treatment of the urine sample (5 mL): calibration curve concentrations = 0, 5, 10, and 50 µg/L; acidification with HCl; centrifugation (10 minutes at 3500 rpm); purification on SPE (Isolute C18 500 mg/3 mL). Chromatographic conditions: Mobile Phase = 60% acetic acid 1% and 40% methanol; Flow = 0.3 mL/min; Column = Symmetry C18 3.0 x 150 mm, 3.5 µm (Waters); Column temperature = 29°C; Run time = 45 min; Volume injected = 21 µL; MS Method = Single Ion Recording of mass 238.0 in ESI neg; LR = 0.3 µg/L.

MA. Pre-treatment of the urine sample (2 mL): calibration curve concentrations: 0, 50, 200, 500, 1000 µg/L; centrifugation (10 minutes at 3500 rpm); purification on SPE (Isolute SAX 500 mg/3mL). Chromatographic conditions: Mobile Phase = 78 % formic acid 0.2 % and 22 % methanol; Flow = 0.3 mL/min; Column= Symmetry C18 3.0 x 150 mm, 3.5 µm (Waters); Column temperature = 30°C; Run time = 30 min; Volume injected = 21 µL. MS Method: Single Ion Recording of mass 141.0 in ESI neg; LR = 7 µg/L.

Cotinine. Pre-treatment of the urine sample (2 mL): calibration curve concentrations: 0, 10, 50, 250, 1000, 3000 µg/L; basification with Ammonium Hydroxide ACS Reagent; centrifugation (10 minutes at 3500 rpm); purification on SPE (Isolute ENV + 50 mg/3mL). Chromatographic conditions: Mobile Phase = 75 % ammonium acetate 3.7mM and 25 % methanol; Flow = 0.3 mL/min; Column = Symmetry C18 3.0 x 150 mm, 3.5 µm (Waters); Column temperature = 30°C; Run time = 33 min; Volume of sample injected = 21 µL. MS Method: Single Ion Recording of mass 177.2 in ESI pos; LR = 0.3 µg/L.

Appendix Table A. Urinary cotinine levels (ln µg/g of creatinine) by smoking habits of the parents, gender, age, season, province of residence, and caseness (295 observations from 95 children)

	β	95% CI (β)	p(Z)
Parental smoking habits	Reference Nonsmokers		
One parent smoking	0.852	0.50; 1.20	<0.001
Both parents smoking	1.685	1.22; 2.15	<0.001
Gender (male vs female)	0.028	-0.31; 0.37	0.872
Age (at the benzene survey)	Reference [6-12] years		
[2-4) years	0.214	-0.22; 0.65	0.338
[4-6) years	0.111	-0.27; 0.49	0.566
Season	Reference Spring		
Summer	-0.193	-0.43; 0.05	0.116
Autumn	-0.015	-0.26; 0.23	0.901
Winter	0.260	0.02; 0.50	0.035
Residence	Reference Turin		
Milan	-0.348	-0.90; 0.20	0.215
Florence - Rome	0.636	0.14; 1.13	0.011
Catania - Palermo - Cagliari	0.511	0.002; 1.02	0.049
Case vs control	0.229	-0.09; 0.55	0.164
R^2 overall =0.4213 (within = 0.0732; between = 0.5150); Wald χ^2=110.31; p<0.0001			

Appendix Table B. Participation bias factors calculated using different cut-points to dicothomize outdoor benzene concentrations

Cut-point = P50 = 3.25 µg/m^3		Exposed	Not Exposed	Bias factor
Cases	Participant	18	25	1.03
	Non Participant	11	12	
Controls	Participant	28	28	
	Non Participant	44	36	
Cut-point = P75 = 4.34 µg/m^3		Exposed	Not Exposed	Bias factor
Cases	Participant	4	39	0.64
	Non Participant	7	16	
Controls	Participant	14	42	
	Non Participant	26	54	
Cut-point = 5 µg/m^3		Exposed	Not Exposed	Bias factor
Cases	Participant	3	40	1.42
	Non Participant	4	19	
Controls	Participant	4	52	
	Non Participant	16	64	

Appendix Table C. Relationship between estimated exposures to ELF-MF (48 h TWA in the child's bedroom, ln μT) and to outdoor benzene (individual averages of repeated seasonal measurements, μg/m^3), controlling for gender, age, province of residence, caseness, and participation in the benzene pilot study (125 observations; 48 cases and 77 controls)

	β	95% CI (β)	p (t)
Outdoor benzene (μg/m^3)	0.177	0.06; 0.29	0.002
Gender (male vs female)	-0.332	-0.74; 0.08	0.112
Age (at diagnosis)	Reference [6-10] years		
[0-2) years	0.120	-0.56; 0.80	0.728
[2-4) years	0.166	-0.38; 0.72	0.550
[4-6) years	0.334	-0.29; 0.96	0.295
Residence	Reference Turin		
Milan	-0.007	-0.65; 0.64	0.984
Florence-Rome	0.135	-0.50; 0.76	0.673
Catania-Palermo-Cagliari	0.521	-0.13; 1.17	0.116
Case vs control	-0.024	-0.43; 0.38	0.908
Participant vs non participant	0.520	0.09; 0.95	0.019
F (10, 114 df) = 2.13; prob > F = 0.0271; R^2 = 0.1577			

STROBE Statement—Checklist of items that should be included in reports of ***case-control studies***

	Item No	Recommendation
Title and abstract	1★	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2★	Explain the scientific background and rationale for the investigation being reported
Objectives	3★	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4★	Present key elements of study design early in the paper
Setting	5★	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6★	(*a*) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls
		(*b*) For matched studies, give matching criteria and the number of controls per case
Variables	7★	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*★	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9★	Describe any efforts to address potential sources of bias
Study size	10★	Explain how the study size was arrived at
Quantitative variables	11★	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12★	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, explain how matching of cases and controls was addressed
		(*e*) Describe any sensitivity analyses
Results		
Participants	13*★	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*★	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
Outcome data	15*★	Report numbers in each exposure category, or summary measures of exposure
Main results	16★	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period

Other analyses	17★	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18★	Summarise key results with reference to study objectives
Limitations	19★	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20★	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21★	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22★	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.